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(54) Title: CORYNEFORM BACTERIA WHICH PRODUCE CHEMICAL COMPOUNDS I

(57) Abstract: The invention relates to coryneform bacteria which have, in addition to at least one copy, present at the natural site (locus), of an open reading frame (ORF), gene or allele which codes for the synthesis of a protein or an RNA, in each case a second, optionally third or fourth copy of this open reading frame (ORF), gene or allele at in each case a second, optionally third or fourth site in a form integrated into the chromosome and processes for the preparation of chemical compounds by fermentation of these bacteria.

Coryneform Bacteria which Produce Chemical Compounds I

Prior Art

Chemical compounds, which means, in particular, L-amino acids, vitamins, nucleosides and nucleotides and D-amino acids, are used in human medicine, in the pharmaceuticals industry, in cosmetics, in the foodstuffs industry and in animal nutrition.

Numerous of these compounds are prepared by fermentation from strains of coryneform bacteria, in particular Corynebacterium glutamicum. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and which produce the particular compounds are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of Corynebacterium strains, by amplifying individual biosynthesis genes and investigating the effect on production.

A common method comprises amplification of certain biosynthesis genes in the particular microorganism by means of episomally replicating plasmids. This procedure has the

disadvantage that during the fermentation, which in industrial processes is in general associated with numerous generations, the plasmids are lost spontaneously (segregational instability).

Another method comprises duplicating certain biosynthesis genes by means of plasmids which do not replicate in the particular microorganism. In this method, the plasmid, including the cloned biosynthesis gene, is integrated into the chromosomal biosynthesis gene of the microorganism (Reinscheid et al., Applied and Environmental Microbiology 60(1), 126-132 (1994); Jetten et al., Applied Microbiology and Biotechnology 43(1):76-82 (1995)). A disadvantage of this method is that the nucleotide sequences of the plasmid and of the antibiotic resistance gene necessary for the selection remain in the microorganism. This is a disadvantage, for example, for the disposal and utilization of the biomass. Moreover, the expert expects such strains to be unstable as a result of disintegration by "Campbell type cross over" in a corresponding number of generations such as are usual in industrial fermentations.

Object of the Invention

The inventors had the object of providing new measures for improved fermentative preparation chemical compounds using coryneform bacteria.

Summary of the Invention

Coryneform bacteria which produce chemical compounds, characterised in that these have, in addition to at least one copy, present at the natural site (locus), of an open reading frame (ORF), gene or allele which codes for the synthesis of a protein or an RNA, a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at a second, optionally third or fourth site in a form integrated into the chromosome, no

nucleotide sequence which is capable of/enables episomal replication or transposition in microorganisms and no nucleotide sequence(s) which impart(s) resistance to antibiotics being present at the second, optionally third or fourth site, and the second, optionally third or fourth site not relating to open reading frames (ORF), genes or alleles which are essential for the growth of the bacteria and the production of the desired compound.

The invention also provides processes for the preparation of one or more chemical compounds, in which the following steps are carried out:

a) fermentation of coryneform bacteria,

a1) which have, in addition to at least one copy, present at the natural site (locus), of an open reading frame (ORF), gene or allele which codes for the synthesis of a protein or an RNA, a second, optionally third or fourth copy of this open reading frame (ORF), gene or allele at a second, optionally third or fourth site in a form integrated into the chromosome, no nucleotide sequence which is capable of/enables episomal replication or transposition in microorganisms and no nucleotide sequence(s) which impart(s) resistance to antibiotics being present at the second, optionally third or fourth site, and the second, optionally third or fourth site not relating to open reading frames (ORF), genes or alleles which are essential for the growth of the bacteria and the production of the desired compound, and

a2) in which the intracellular activity of the corresponding protein is increased, in particular the nucleotide sequence which codes for this protein is over-expressed,

b) concentration of the chemical compound(s) in the fermentation broth and/or in the cells of the bacteria,

c) isolation of the chemical compound(s), optionally

d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100 wt.%.

The invention also provides processes for the preparation of one or more chemical compounds, which comprise the following steps:

a) fermentation of coryneform bacteria, in particular of the genus Corynebacterium, which have, in addition to the copy of an open reading frame (ORF), gene or allele present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at in each case a second, optionally third or fourth site in integrated form, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular second, optionally third or fourth site,

under conditions which allow expression of the said open reading frames (ORF), genes or alleles

b) concentration of the chemical compound(s) in the fermentation broth and/or in the cells of the bacteria,

c) isolation of the chemical compound(s), optionally

d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

Detailed Description of the Invention

Chemical compounds are to be understood, in particular, as meaning amino acids, vitamins, nucleosides and nucleotides. The biosynthesis pathways of these compounds are known and are available in the prior art.

Amino acids mean, preferably, L-amino acids, in particular the proteinogenic L-amino acids, chosen from the group consisting of L-aspartic acid, L-asparagine, L-threonine, L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and L-arginine and salts thereof, in particular L-lysine, L-methionine and L-threonine. L-Lysine is very particularly preferred.

Proteinogenic amino acids are understood as meaning the amino acids which occur in natural proteins, that is to say in proteins of microorganisms, plants, animals and humans.

Vitamins mean, in particular, vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxines), vitamin B12 (cyanocobalamin), nicotinic acid/nicotinamide, vitamin M (folic acid) and vitamin E (tocopherol) and salts thereof, pantothenic acid being preferred.

Nucleosides and nucleotides mean, inter alia, S-adenosyl-methionine, inosine-5'-monophosphoric acid and guanosine-5'-monophosphoric acid and salts thereof.

The coryneform bacteria are, in particular, those of the genus Corynebacterium. Of the genus Corynebacterium, the species Corynebacterium glutamicum, Corynebacterium

ammoniagenes and Corynebacterium thermoaminogenes are preferred. Information on the taxonomic classification of strains of this group of bacteria is to be found, inter alia, in Kämpfer and Kroppenstedt (Canadian Journal of Microbiology 42, 989-1005 (1996)) and in US-A-5,250,434.

Suitable strains of the species Corynebacterium glutamicum (C. glutamicum) are, in particular, the known wild-type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium lilium ATCC15990
Corynebacterium melassecola ATCC17965
Corynebacterium herculis ATCC13868
Arthrobacter sp. ATCC243
Brevibacterium chang-fua ATCC14017
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869
Brevibacterium divaricatum ATCC14020
Brevibacterium taipei ATCC13744 and
Microbacterium ammoniaphilum ATCC21645

and mutants or strains, such as are known from the prior art, produced therefrom which produce chemical compounds.

Suitable strains of the species Corynebacterium ammoniagenes (C. ammoniagenes) are, in particular, the known wild-type strains

Brevibacterium ammoniagenes ATCC6871
Brevibacterium ammoniagenes ATCC15137 and
Corynebacterium sp. ATCC21084

and mutants or strains, such as are known from the prior art, produced therefrom which produce chemical compounds.

Suitable strains of the species Corynebacterium thermoaminogenes (C. thermoaminogenes) are, in particular, the known wild-type strains

Corynebacterium thermoaminogenes FERM BP-1539
Corynebacterium thermoaminogenes FERM BP-1540
Corynebacterium thermoaminogenes FERM BP-1541 and
Corynebacterium thermoaminogenes FERM BP-1542

and mutants or strains, such as are known from the prior art, produced therefrom which produce chemical compounds.

Strains with the designation "ATCC" can be obtained from the American Type Culture Collection (Manassas, VA, USA). Strains with the designation "FERM" can be obtained from the National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba Ibaraki, Japan). The strains of Corynebacterium thermoaminogenes mentioned (FERM BP-1539, FERM BP-1540, FERM BP-1541 and FERM BP-1542) are described in US-A 5,250,434.

Open reading frame (ORF) describes a section of a nucleotide sequence which codes or can code for a protein or polypeptide or ribonucleic acid to which no function can be assigned according to the prior art.

After assignment of a function to the nucleotide sequence section in question, it is in general referred to as a gene.

Alleles are in general understood as meaning alternative forms of a given gene. The forms are distinguished by differences in the nucleotide sequence.

In the context of the present invention, endogenous, that is to say species-characteristic, open reading frames, genes or alleles are preferably used. These are understood as meaning the open reading frames, genes or alleles or

nucleotide sequences thereof present in the population of a species, such as, for example, Corynebacterium glutamicum.

"A copy of an open reading frame (ORF), a gene or allele present at the natural site (locus)" in the context of this invention is understood as meaning the position or situation of the ORF or gene or allele in relation to the adjacent ORFs or genes or alleles such as exists in the corresponding wild-type or corresponding parent organism or starting organism.

Thus, for example, the natural site of the lysC gene or of an $lysC^{FBR}$ allele, which codes for a "feed back" resistant aspartate kinase from Corynebacterium glutamicum is the lysC site or lysC locus or lysC gene site with the directly adjacent genes or open reading frames orfX and leuA on one flank and the asd gene on the other flank.

"Feed back" resistant aspartate kinase is understood as meaning aspartate kinases which, compared with the wild-type form, have a lower sensitivity to inhibition by mixtures of lysine and threonine or mixtures of AEC (aminoethylcysteine) and threonine or lysine by itself or AEC by itself. Strains which produce L-lysine typically contain such "feed back" resistant or desensitized aspartate kinases.

The nucleotide sequence of the chromosome of Corynebacterium glutamicum is known and can be found in Patent Application EP-A-1108790 and Access Number (Accession No.) AX114121 of the nucleotide sequence databank of the European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany and Cambridge, UK). The nucleotide sequences of orfX, the leuA gene and the asd gene have the Access Numbers AX120364 (orfX), AX123517 (leuA) and AX123519 (asd).

Further databanks, such as, for example, that of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) or that of the Swiss Institute of Bioinformatics (Swissprot, Geneva, Switzerland) or that of the Protein Information Resource Database (PIR, Washington, DC, USA) can also be used.

"In each case a second, optionally third or fourth site" is understood as meaning a site which differs from the "natural site". It is also called a "target site" or "target sequence" in the following. It can also be called an "integration site" or "transformation site". This second, optionally third or fourth site, or the nucleotide sequence present at the corresponding sites, is preferably in the chromosome and is in general not essential for growth and for production of the desired chemical compounds.

To produce the coryneform bacteria according to the invention, the nucleotide sequence of the desired ORF, gene or allele, optionally including expression and/or regulation signals, is isolated and provided with nucleotide sequences of the target site at the ends, these are then transferred into the desired coryneform bacterium, preferably with the aid of vectors which do not replicate or replicate to only a limited extent in coryneform bacteria, and those bacteria in which the desired ORF, gene or allele is incorporated at the target site are isolated, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the target site.

The invention accordingly also provides a process for the production of coryneform bacteria which produce one or more chemical compounds, which comprises

a) isolating the nucleotide sequence of at least one desired ORF, gene or allele, optionally including the expression and/or regulation signals,

b) providing the 5' and the 3' end of the ORF, gene or allele with nucleotide sequences of the target site,

c) preferably incorporating the nucleotide sequence of the desired ORF, gene or allele provided with nucleotide sequences of the target site into a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,

d) transferring the nucleotide sequence according to b) or c) into coryneform bacteria, and

e) isolating coryneform bacteria in which the nucleotide sequence according to a) is incorporated at the target site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the target site.

Preferably, also, no residues of sequences of the vectors used or species-foreign DNA, such as, for example, restriction cleavage sites, remain at the target site. A maximum of 24, preferably a maximum of 12, particularly preferably a maximum of 6 nucleotides of such DNA upstream or downstream of the ORF, gene or allele incorporated optionally remain at the target site.

By the measures according to the invention, the productivity of the coryneform bacteria or of the fermentative processes for the preparation of chemical compounds is improved in respect of one or more of the features chosen from the group consisting of concentration (chemical compound formed, based on the unit volume), yield

(chemical compound formed, based on the source of carbon consumed) and product formation rate (chemical compound formed, based on the time) by at least 0.5 - 1.0% or at least 1.0 to 1.5% or at least 1.5 - 2.0%.

Instructions on conventional genetic engineering methods, such as, for example, isolation of chromosomal DNA, plasmid DNA, handling of restriction enzymes etc., are found in Sambrook et al. (Molecular Cloning - A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press). Instructions on transformation and conjugation in coryneform bacteria are found, inter alia, in Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), in Schäfer et al. (Journal of Bacteriology 172, 1663-1666 (1990) and Gene 145, 69-73 (1994)) and in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)).

Vectors which replicate to only a limited extent are understood as meaning plasmid vectors which, as a function of the conditions under which the host or carrier is cultured, replicate or do not replicate. Thus, a temperature-sensitive plasmid for coryneform bacteria which can replicate only at temperatures below 31ºC has been described by Nakamura et al. (US-A-6,303,383).

The invention furthermore provides coryneform bacteria, in particular of the genus Corynebacterium, which produce L-lysine, characterized in that these have, in addition to at least one of the copy of an open reading frame (ORF), gene or allele of lysine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at in each case a second, optionally third or fourth site in integrated form, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which

imparts resistance to antibiotics being present at the particular second, optionally third or fourth site.

The invention also furthermore provides a process for the preparation of L-lysine, which comprises the following steps:

a) fermentation of coryneform bacteria, in particular Corynebacterium glutamicum, characterized in that these have, in addition to at least one of the copy of an open reading frame (ORF), gene or allele of lysine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at in each case a second, optionally third or fourth site in integrated form, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular second, optionally third or fourth site,

under conditions which allow expression of the said open reading frames (ORF), genes or alleles,

b) concentration of the L-lysine in the fermentation broth,

c) isolation of the L-lysine from the fermentation broth, optionally

d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

A "copy of an open reading frame (ORF), gene or allele of lysine production" is to be understood as meaning all the, preferably endogenous, open reading frames, genes or

alleles of which enhancement/over-expression can have the effect of improving lysine production. Enhancement is understood as meaning an increase in the intracellular concentration or activity of the particular gene product, protein or enzyme.

These include, inter alia, the following open reading frames, genes or alleles: accBC, accDA, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dapA, dapB, dapC, dapD, dapE, dapF, ddh, dps, eno, gap, gap2, gdh, gnd, lysC, $lysC^{FBR}$, lysE, msiK, opcA, oxyR, ppc, ppc^{FBR}, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T. These are summarized and explained in Table 1.

These include, in particular, the $lysC^{FBR}$ alleles which code for a "feed back" resistant aspartate kinase. Various $lysC^{FBR}$ alleles are summarized and explained in Table 2.

The following $lysC^{FBR}$ alleles are preferred: lysC A279T (replacement of alanine at position 279 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by threonine), lysC A279V (replacement of alanine at position 279 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by valine), lysC S301F (replacement of serine at position 301 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by phenylalanine), lysC T308I (replacement of threonine at position 308 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by isoleucine), lysC S301Y (replacement of serine at position 308 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by tyrosine), lysC G345D (replacement of glycine at position 345 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by aspartic acid), lysC R320G (replacement of arginine at position 320 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by glycine), lysC T311I (replacement of threonine at position 311 of the aspartate kinase protein coded, according to SEQ

ID NO: 2, by isoleucine), lysC S381F (replacement of serine at position 381 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by phenylalanine).

The $lysC^{FBR}$ allele lysC T311I (replacement of threonine at position 311 of the aspartate kinase protein coded, according to SEQ ID NO: 2, by isoleucine), the nucleotide sequence of which is shown as SEQ ID NO:3, is particularly preferred; the amino acid sequence of the aspartate kinase protein coded is shown as SEQ ID NO:4.

The second, optionally third or fourth copy of the open reading frame (ORF), gene or allele of lysine production in question can be integrated at in each case a second, optionally third or fourth site. The following open reading frames, genes or nucleotide sequences, inter alia, can be used for this: aecD, ccpA1, ccpA2, citA, citB, citE, fda, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, mqo, pck, pgi, poxB and zwa2, in particular the genes aecD, gluA, gluB, gluC, gluD and pck. These are summarized and explained in Table 3.

The sites mentioned include, of course, not only the coding regions of the open reading frames or genes mentioned, but also the regions or nucleotide sequences lying upstream which are responsible for expression and regulation, such as, for example, ribosome binding sites, promoters, binding sites for regulatory proteins, binding sites for regulatory ribonucleic acids and attenuators. These regions in general lie in a range of 1-800, 1-600, 1-400, 1-200, 1-100 or 1-50 nucleotides upstream of the coding region. In the same way, regions lying downstream, such as, for example, transcription terminators, are also included. These regions in general lie in a range of 1-400, 1-200, 1-100, 1-50 or 1-25 nucleotides downstream of the coding region.

Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can

furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

A prophage is understood as meaning a bacteriophage, in particular the genome thereof, where this is replicated together with the genome of the host and the formation of infectious particles does not take place. A defective phage is understood as meaning a prophage, in particular the genome thereof, which, as a result of various mutations, has lost the ability to form so-called infectious particles. Defective phages are also called cryptic. Prophages and defective phages are often present in integrated form in the chromosome of their host. Further details exist in the prior art, for example in the textbook by Edward A. Birge (Bacterial and Bacteriophage Genetics, 3rd ed., Springer-Verlag, New York, USA, 1994) or in the textbook by S. Klaus et al. (Bakterienviren, Gustav Fischer Verlag, Jena, Germany, 1992).

Table 1

Open reading frames, genes and alleles of lysine production

Name	Description of the coded enzyme or protein	Reference	Access Number
accBC	Acyl-CoA Carboxylase EC 6.3.4.14 (acyl-CoA carboxylase)	Jäger et al. Archives of Microbiology (1996) 166:76-82 EP1108790; WO0100805	U35023 AX123524 AX066441
accDA	Acetyl-CoA Carboxylase EC 6.4.1.2 (acetyl-CoA carboxylase)	EP1055725 EP1108790 WO0100805	 AX121013 AX066443
cstA	Carbon Starvation Protein A (carbon starvation protein A)	EP1108790 WO0100804	AX120811 AX066109
cysD	Sulfate Adenylyltransferase sub-unit II EC 2.7.7.4 (sulfate adenylyltransferase small chain)	EP1108790	AX123177
cysE	Serine Acetyltransferase EC 2.3.1.30 (serine acetyltransferase)	EP1108790 WO0100843	AX122902 AX063961
cysH	3'-Phosphoadenyl Sulfate Reductase EC 1.8.99.4 (3'-phosphoadenosine 5'-phosphosulfate reductase)	EP1108790 WO0100842	AX123178 AX066001
cysK	Cysteine Synthase EC 4.2.99.8 (cysteine synthase)	EP1108790 WO0100843	AX122901 AX063963
cysN	Sulfate Adenylyltransferase sub-unit I EC 2.7.7.4 (sulfate adenylyltransferase)	EP1108790	AX123176 AX127152
cysQ	Transport Protein CysQ (transporter cysQ)	EP1108790 WO0100805	AX127145 AX066423
dapA	Dihydrodipicolinate Synthase EC 4.2.1.52 (dihydrodipicolinate synthase)	Bonnassie et al. Nucleic Acids Research 18:6421 (1990) Pisabarro et al., Journal of Bacteriology 175:2743-2749(1993) EP1108790 WO0100805 EP0435132 EP1067192 EP1067193	X53993 Z21502 AX123560 AX063773
dapB	Dihydrodipicolinate Reductase EC 1.3.1.26 (dihydrodipicolinate reductase)	EP1108790 WO0100843 EP1067192	AX127149 AX063753 AX137723

		EP1067193 Pisabarro et al., Journal of Bacteriology 175:2743-2749(1993) JP1998215883 JP1997322774 JP1997070291 JP1995075578	AX137602 X67737 Z21502 E16749 E14520 E12773 E08900
dapC	N-Succinyl Aminoketopimelate Transaminase EC 2.6.1.17 (N-succinyl diaminopimelate transaminase)	EP1108790 WO0100843 EP1136559	AX127146 AX064219
dapD	Tetrahydrodipicolinate Succinylase EC 2.3.1.117 (tetrahydrodipicolinate succinylase)	EP1108790 WO0100843 Wehrmann et al. Journal of Bacteriology 180:3159-3165(1998)	AX127146 AX063757 AJ004934
dapE	N-Succinyl Diaminopimelate Desuccinylase EC 3.5.1.18 (N-succinyl diaminopimelate desuccinylase)	EP1108790 WO0100843 Wehrmann et al. Microbiology 140:3349-3356 (1994)	AX127146 AX063749 X81379
dapF	Diaminopimelate Epimerase EC 5.1.1.7 (diaminopimelate epimerase)	EP1108790 WO0100843 EP1085094	AX127149 AX063719 AX137620
ddh	Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase)	EP1108790 WO0100843 Ishino et al., Nucleic Acids Research 15:3917-3917(1987) JP1997322774 JP1993284970 Kim et al., Journal of Microbiology and Biotechnology 5:250-256(1995)	AX127152 AX063759 Y00151 E14511 E05776 D87976
dps	DNA Protection Protein (protection during starvation protein)	EP1108790	AX127153
eno	Enolase EC 4.2.1.11 (enolase)	EP1108790 WO0100844 EP1090998 Hermann et al., Electrophoresis 19:3217-3221 (1998)	AX127146 AX064945 AX136862
gap	Glyceraldehyde 3-Phosphate Dehydrogenase	EP1108790 WO0100844	AX127148 AX064941

	EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase)	Eikmanns et al., Journal of Bacteriology 174:6076-6086(1992)	X59403
gap2	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase 2)	EP1108790 WO0100844	AX127146 AX064939
gdh	Glutamate Dehydrogenase EC 1.4.1.4 (glutamate dehydrogenase)	EP1108790 WO0100844 Boermann et al., Molecular Microbiology 6:317-326 (1992). Guyonvarch et al., NCBI	AX127150 AX063811 X59404 X72855
gnd	6-Phosphogluconate Dehydrogenase EC 1.1.1.44 (6-phosphogluconate dehydrogenase)	EP1108790 WO0100844	AX127147 AX121689 AX065125
lysC	Aspartate Kinase EC 2.7.2.4 (aspartate kinase)	EP1108790 WO0100844 Kalinowski et al., Molecular Microbiology 5:1197-204 (1991)	AX120365 AX063743 X57226
$lysC^{FBR}$	Aspartate Kinase feedback resistant (fbr) EC 2.7.2.4 (aspartate kinase fbr)	see Table 2	
lysE	Lysine Exporter (lysine exporter protein)	EP1108790 WO0100843 Vrljić et al., Molecular Microbiology 22:815-826 (1996)	AX123539 AX123539 X96471
msiK	Sugar Importer (multiple sugar import protein)	EP1108790	AX120892
opcA	Glucose 6-phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase)	WO0104325	AX076272
oxyR	Transcription Regulator (transcriptional regulator)	EP1108790	AX122198 AX127149
ppc^{FBR}	Phosphoenol Pyruvate Carboxylase feedback resistant EC 4.1.1.31 (phosphoenol pyruvate carboxylase feedback resistant)	EP0723011 WO0100852	
ppc	Phosphoenol Pyruvate Carboxylase EC 4.1.1.31 (phosphoenol pyruvate carboxylase)	EP1108790 O'Reagan et al., Gene 77(2):237-251(1989)	AX127148 AX123554 M25819

pgk	Phosphoglycerate Kinase EC 2.7.2.3 (phosphoglycerate kinase)	EP1108790 WO0100844 Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	AX121838 AX127148 AX064943 X59403
pknA	Protein Kinase A (protein kinase A)	EP1108790	AX120131 AX120085
pknB	Protein Kinase B (protein kinase B)	EP1108790	AX120130 AX120085
pknD	Protein Kinase D (protein kinase D)	EP1108790	AX127150 AX122469 AX122468
pknG	Protein Kinase G (protein kinase G)	EP1108790	AX127152 AX123109
ppsA	Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase)	EP1108790	AX127144 AX120700 AX122469
ptsH	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H)	EP1108790 WO0100844	AX122210 AX127149 AX069154
ptsI	Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I)	EP1108790	AX122206 AX127149
ptsM	Glukose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase system enzyme II)	Lee et al., FEMS Microbiology Letters 119 (1-2):137-145 (1994)	L18874
pyc	Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase)	WO9918228 Peters-Wendisch et al., Microbiology 144:915-927 (1998)	A97276 Y09548
pyc P458S	Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S	EP1108790	
sigC	Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C)	EP1108790	AX120368 AX120085
sigD	RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	EP1108790	AX120753 AX127144
sigE	Sigma Factor E EC 2.7.7.6 (extracytoplasmic function alternative sigma factor E)	EP1108790	AX127146 AX121325
sigH	Sigma Factor H EC 2.7.7.6 (sigma factor SigH)	EP1108790	AX127145 AX120939

sigM	Sigma Factor M EC 2.7.7.6 (sigma factor SigM)	EP1108790	AX123500 AX127145
tal	Transaldolase EC 2.2.1.2 (transaldolase)	WO0104325	AX076272
thyA	Thymidylate Synthase EC 2.1.1.45 (thymidylate synthase)	EP1108790	AX121026 AX127145
tkt	Transketolase EC 2.2.1.1 (transketolase)	Ikeda et al., NCBI	AB023377
tpi	Triose Phosphate Isomerase EC 5.3.1.1 (triose phosphate isomerase)	Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	X59403
zwa1	Cell Growth Factor 1 (growth factor 1)	EP1111062	AX133781
zwf	Glucose 6-phosphate 1-Dehydrogenase EC 1.1.1.49 (glucose 6-phosphate 1-dehydrogenase)	EP1108790 WO0104325	AX127148 AX121827 AX076272
zwf A213T	Glucose 6-phosphate 1-Dehydrogenase EC 1.1.1.49 (glucose 6-phosphate 1-dehydrogenase) amino acid exchange A213T	EP1108790	

Table 2

$lysC^{FBR}$ alleles which code for feed back resistant aspartate kinases

Name of the allele	Further information	Reference	Access Number
$lysC^{FBR}$-E05108		JP 1993184366-A (sequence 1)	E05108
$lysC^{FBR}$-E06825	lysC A279T	JP 1994062866-A (sequence 1)	E06825
$lysC^{FBR}$-E06826	lysC A279T	JP 1994062866-A (sequence 2)	E06826
$lysC^{FBR}$-E06827		JP 1994062866-A (sequence 3)	E06827
$lysC^{FBR}$-E08177		JP 1994261766-A (sequence 1)	E08177
$lysC^{FBR}$-E08178	lysC A279T	JP 1994261766-A (sequence 2)	E08178
$lysC^{FBR}$-E08179	lysC A279V	JP 1994261766-A (sequence 3)	E08179
$lysC^{FBR}$-E08180	lysC S301F	JP 1994261766-A (sequence 4)	E08180
$lysC^{FBR}$-E08181	lysC T308I	JP 1994261766-A (sequence 5)	E08181
$lysC^{FBR}$-E08182		JP 1994261766-A (sequence 6)	E08182
$lysC^{FBR}$-E12770		JP 1997070291-A (sequence 13)	E12770
$lysC^{FBR}$-E14514		JP 1997322774-A (sequence 9)	E14514
$lysC^{FBR}$-E16352		JP 1998165180-A (sequence 3)	E16352
$lysC^{FBR}$-E16745		JP 1998215883-A (sequence 3)	E16745
$lysC^{FBR}$-E16746		JP 1998215883-A (sequence 4)	E16746
$lysC^{FBR}$-I74588		US 5688671-A (sequence 1)	I74588
$lysC^{FBR}$-I74589	lysC A279T	US 5688671-A (sequence 2)	I74589
$lysC^{FBR}$-I74590		US 5688671-A (sequence 7)	I74590
$lysC^{FBR}$-I74591	lysC A279T	US 5688671-A (sequence 8)	I74591
$lysC^{FBR}$-I74592		US 5688671-A (sequence 9)	I74592
$lysC^{FBR}$-I74593	lysC A279T	US 5688671-A (sequence 10)	I74593
$lysC^{FBR}$-I74594		US 5688671-A (sequence 11)	I74594
$lysC^{FBR}$-I74595	lysC A279T	US 5688671-A (sequence 12)	I74595
$lysC^{FBR}$-I74596		US 5688671-A (sequence 13)	I74596

$lysC^{FBR}$-I74597	lysC A279T	US 5688671-A (sequence 14)	I74597
$lysC^{FBR}$-X57226	lysC S301Y	EP0387527 Kalinowski et al., Molecular and General Genetics 224:317-324 (1990)	X57226
$lysC^{FBR}$-L16848	lysC G345D	Follettie and Sinskey NCBI Nucleotide Database (1990)	L16848
$lysC^{FBR}$-L27125	lysC R320G lysC G345D	Jetten et al., Applied Microbiology Biotechnology 43:76-82 (1995)	L27125
$lysC^{FBR}$	lysC T311I	WO0063388 (sequence 17)	
$lysC^{FBR}$	lysC S301F	US3732144	
$lysC^{FBR}$	lysC S381F		
$lysC^{FBR}$		JP6261766 (sequence 1)	
$lysC^{FBR}$	lysC A279T	JP6261766 (sequence 2)	
$lysC^{FBR}$	lysC A279V	JP6261766 (sequence 3)	
$lysC^{FBR}$	lysC S301F	JP6261766 (sequence 4)	
$lysC^{FBR}$	lysC T308I	JP6261766 (sequence 5)	

Table 3

Target sites for integration of open reading frames, genes and alleles of lysine production

Gene name	Description of the coded enzyme or protein	Reference	Access Number
aecD	beta C-S Lyase EC 2.6.1.1 (beta C-S lyase)	Rossol et al., Journal of Bacteriology 174(9):2968-77 (1992)	M89931
ccpA1	Catabolite Control Protein (catabolite control protein A1)	WO0100844 EP1108790	AX065267 AX127147
ccpA2	Catabolite Control Protein (catabolite control protein A2)	WO0100844 EP1108790	AX065267 AX121594
citA	Sensor Kinase CitA (sensor kinase CitA)	EP1108790	AX120161
citB	Transcription Regulator CitB (transcription regulator CitB)	EP1108790	AX120163
citE	Citrate Lyase EC 4.1.3.6 (citrate lyase)	WO0100844 EP1108790	AX065421 AX127146
fda	Fructose Bisphosphate Aldolase EC 4.1.2.13 (fructose 1,6-bisphosphate aldolase)	von der Osten et al., Molecular Microbiology 3(11):1625-37 (1989)	X17313
gluA	Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluB	Glutamate-binding Protein (glutamate-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluC	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluD	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
luxR	Transcription Regulator LuxR (transcription regulator LuxR)	WO0100842 EP1108790	AX065953 AX123320
luxS	Histidine Kinase LuxS (histidine kinase LuxS)	EP1108790	AX123323 AX127145
lysR1	Transcription Regulator LysR1	EP1108790	AX064673 AX127144

	(transcription regulator LysR1)		
lysR2	Transcription Activator LysR2 (transcription regulator LysR2)	EP1108790	AX123312
lysR3	Transcription Regulator LysR3 (transcription regulator LysR3)	WO0100842 EP1108790	AX065957 AX127150
menE	O-Succinylbenzoic Acid CoA Ligase EC 6.2.1.26 (O-succinylbenzoate CoA ligase)	WO0100843 EP1108790	AX064599 AX064193 AX127144
mqo	Malate-Quinone Oxidoreductase (malate-quinone-oxidoreductase)	Molenaar et al., Eur. Journal of Biochemistry 1;254(2):395-403 (1998)	AJ224946
pck	Phosphoenol Pyruvate Carboxykinase (phosphoenol pyruvate carboxykinase)	WO0100844	AJ269506 AX065053
pgi	Glucose 6-phosphate Isomerase EC 5.3.1.9 (glucose 6-phosphate isomerase)	EP1087015 EP1108790	AX136015 AX127146
poxB	Pyruvate Oxidase EC 1.2.3.3 (pyruvate oxidase)	WO0100844 EP1096013	AX064959 AX137665
zwa2	Cell Growth Factor 2 (growth factor 2)	EP1106693 EP1108790	AX113822 AX127146

The invention accordingly also provides a process for the production of coryneform bacteria which produce L-lysine, which comprises

a) isolating the nucleotide sequence of at least one desired ORF, gene or allele of lysine production, optionally including the expression and/or regulation signals,

b) providing the 5' and the 3' end of the ORF, gene or allele of lysine production with nucleotide sequences of the target site,

c) preferably incorporating the nucleotide sequence of the desired ORF, gene or allele provided with

nucleotide sequences of the target site into a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,

d) transferring the nucleotide sequence according to b) or c) into coryneform bacteria, and

e) isolating coryneform bacteria in which the nucleotide sequence according to a) is incorporated at the target site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the target site.

The invention furthermore provides coryneform bacteria, in particular of the genus Corynebacterium, which produce L-methionine and/or L-threonine, characterized in that these have, in addition to at least one of the copy of an open reading frame (ORF), gene or allele of methionine production or threonine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at in each case a second, optionally third or fourth site in integrated form, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular second, optionally third or fourth site.

The invention also furthermore provides a process for the preparation of L-methionine and/or L-threonine, which comprises the following steps:

a) fermentation of coryneform bacteria, in particular Corynebacterium glutamicum, characterized in that

these have, in addition to at least one of the copy of an open reading frame (ORF), gene or allele of methionine production or threonine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at in each case a second, optionally third or fourth site in integrated form, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular second, optionally third or fourth site,

under conditions which allow expression of the said open reading frames (ORF), genes or alleles,

b) concentration of the L-methionine and/or L-threonine in the fermentation broth,

c) isolation of the L-methionine and/or L-threonine from the fermentation broth, optionally

d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

A "copy of an open reading frame (ORF), gene or allele of methionine production" is to be understood as meaning all the, preferably endogenous, open reading frames, genes or alleles of which enhancement/over-expression can have the effect of improving methionine production.

These include, inter alia, the following open reading frames, genes or alleles: accBC, accDA, aecD, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dps, eno, fda, gap, gap2, gdh, gnd, glyA, hom, hom^{FBR}, lysC, $lysC^{FBR}$, metA, metB, metE,

metH, metY, msiK, opcA, oxyR, ppc, ppc^{FBR}, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T. These are summarized and explained in Table 4. These include, in particular, the lysC^{FBR} alleles which code for a "feed back" resistant aspartate kinase (see Table 2) and the hom^{FBR} alleles which code for a "feed back" resistant homoserine dehydrogenase.

The second, optionally third or fourth copy of the open reading frame (ORF), gene or allele of methionine production in question can be integrated at in each case a second, optionally third or fourth site. The following open reading frames, genes or nucleotide sequences, inter alia, can be used for this: brnE, brnF, brnQ, ccpA1, ccpA2, citA, citB, citE, ddh, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, metD, metK, pck, pgi, poxB and zwa2. These are summarized and explained in Table 5.

The sites mentioned include, of course, not only the coding regions of the open reading frames or genes mentioned, but also the regions or nucleotide sequences lying upstream which are responsible for expression and regulation, such as, for example, ribosome binding sites, promoters, binding sites for regulatory proteins, binding sites for regulatory ribonucleic acids and attenuators. These regions in general lie in a range of 1-800, 1-600, 1-400, 1-200, 1-100 or 1-50 nucleotides upstream of the coding region. In the same way, regions lying downstream, such as, for example, transcription terminators, are also included. These regions in general lie in a range of 1-400, 1-200, 1-100, 1-50 or 1-25 nucleotides downstream of the coding region.

Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

Table 4

Open reading frames, genes and alleles of methionine production

Name	Description of the coded enzyme or protein	Reference	Access Number
AccBC	Acyl-CoA Carboxylase EC 6.3.4.14 (acyl-CoA carboxylase)	Jäger et al. Archives of Microbiology (1996) 166:76-82 EP1108790; WO0100805	U35023 AX123524 AX066441
AccDA	Acetyl-CoA Carboxylase EC 6.4.1.2 (acetyl-CoA carboxylase)	EP1055725 EP1108790 WO0100805	 AX121013 AX066443
AecD	Cystathionine beta-Lyase EC 4.4.1.8 (cystathionine beta-lyase)	Rossol et al., Journal of Bacteriology 174:2968-2977 (1992)	M89931
CstA	Carbon Starvation Protein A (carbon starvation protein A)	EP1108790 WO0100804	AX120811 AX066109
CysD	Sulfate Adenylyltransferase sub-unit II EC 2.7.7.4 (sulfate adenylyltransferase small chain)	EP1108790	AX123177
CysE	Serine Acetyltransferase EC 2.3.1.30 (serine acetyltransferase)	EP1108790 WO0100843	AX122902 AX063961
CysH	3'-Phosphoadenyl Sulfate Reductase EC 1.8.99.4 (3'-phosphoadenosine 5'-phosphosulfate reductase)	EP1108790 WO0100842	AX123178 AX066001
CysK	Cysteine Synthase EC 4.2.99.8 (cysteine synthase)	EP1108790 WO0100843	AX122901 AX063963
CysN	Sulfate Adenylyltransferase sub-unit I EC 2.7.7.4 (sulfate adenylyltransferase)	EP1108790	AX123176 AX127152
CysQ	Transport protein CysQ (transporter cysQ)	EP1108790 WO0100805	AX127145 AX066423
Dps	DNA Protection Protein (protection during starvation protein)	EP1108790	AX127153
Eno	Enolase EC 4.2.1.11 (enolase)	EP1108790 WO0100844 EP1090998 Hermann et al., Electrophoresis 19:3217-3221 (1998)	AX127146 AX064945 AX136862

Fda	Fructose Bisphosphate Aldolase EC 4.1.2.13 (fructose bisphosphate aldolase)	van der Osten et al., Molecular Microbiology 3:1625-1637 (1989)	X17313
Gap	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase)	EP1108790 WO0100844 Eikmanns et al., Journal of Bacteriology 174:6076-6086(1992)	AX127148 AX064941 X59403
gap2	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase 2)	EP1108790 WO0100844	AX127146 AX064939
Gdh	Glutamate Dehydrogenase EC 1.4.1.4 (glutamate dehydrogenase)	EP1108790 WO0100844 Boermann et al., Molecular Microbiology 6:317-326 (1992); Guyonvarch et al., NCBI	AX127150 AX063811 X59404 X72855
GlyA	Glycine/Serine Hydroxymethyltransferase EC 2.1.2.1 (glycine/serine hydroxymethyltransferase)	EP1108790	AX127146 AX121194
Gnd	6-Phosphogluconate Dehydrogenase EC 1.1.1.44 (6-phosphogluconate dehydrogenase)	EP1108790 WO0100844	AX127147 AX121689 AX065125
Hom	Homoserine Dehydrogenase EC 1.1.1.3 (homoserine dehydrogenase)	Peoples et al., Molecular Microbiology 2:63-72 (1988)	Y00546
hom^{FBR}	Homoserine Dehydrogenase feedback resistant (fbr) EC 1.1.1.3 (homoserine dehydrogenase fbr)	Reinscheid et al., Journal of Bacteriology 173:3228-30 (1991)	
LysC	Aspartate Kinase EC 2.7.2.4 (aspartate kinase)	EP1108790 WO0100844 Kalinowski et al., Molecular Microbiology 5:1197-204 (1991)	AX120365 AX063743 X57226
$lysC^{FBR}$	Aspartate Kinase feedback resistant (fbr) EC 2.7.2.4 (aspartate kinase fbr)	see Table 2	
MetA	Homoserine Acetyltransferase EC 2.3.1.31 (homoserine acetyltransferase)	Park et al., Molecular Cells 8:286-94 (1998)	AF052652
MetB	Cystathionine γ-Lyase	Hwang et al.,	AF126953

	EC 4.4.1.1 (cystathionine gamma-synthase)	Molecular Cells 9:300-308 (1999)	
MetE	Homocysteine Methyltransferase EC 2.1.1.14 (homocysteine methyltransferase)	EP1108790	AX127146 AX121345
MetH	Homocysteine Methyltransferase (Vitamin B12-dependent) EC 2.1.1.14 (homocysteine methyltransferase)	EP1108790	AX127148 AX121747
MetY	Acetylhomoserine Sulfhydrolase (acetylhomoserine sulfhydrolase)	EP1108790	AX120810 AX127145
MsiK	Sugar Importer (multiple sugar import protein)	EP1108790	AX120892
OpcA	Glucose 6-phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase)	WO0104325	AX076272
OxyR	Transcription Regulator (transcriptional regulator)	EP1108790	AX122198 AX127149
ppc^{FBR}	Phosphoenol Pyruvate Carboxylase feedback resistent EC 4.1.1.31 (phosphoenol pyruvate carboxylase feedback resistant)	EP0723011 WO0100852	
Ppc	Phosphoenol Pyruvate Carboxylase EC 4.1.1.31 (phosphoenol pyruvate carboxylase)	EP1108790 O'Reagan et al., Gene 77(2):237-251(1989)	AX127148 AX123554 M25819
Pgk	*Phosphoglycerate Kinase* EC 2.7.2.3 (phosphoglycerate kinase)	EP1108790 WO0100844 Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	AX121838 AX127148 AX064943 X59403
PknA	Protein Kinase A (protein kinase A)	EP1108790	AX120131 AX120085
PknB	Protein Kinase B (protein kinase B)	EP1108790	AX120130 AX120085
PknD	Protein Kinase D (protein Kinase D)	EP1108790	AX127150 AX122469 AX122468
PknG	Protein Kinase G (protein kinase G)	EP1108790	AX127152 AX123109
PpsA	Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase)	EP1108790	AX127144 AX120700 AX122469
PtsH	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H)	EP1108790 WO0100844	AX122210 AX127149 AX069154
PtsI	Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I)	EP1108790	AX122206 AX127149
PtsM	Glucose-specific Phosphotransferase System Enzyme II	Lee et al., FEMS Microbiology	L18874

	EC 2.7.1.69 (glucose phosphotransferase system enzyme II)	Letters 119 (1-2):137-145 (1994)	
Pyc	Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase)	WO9918228 Peters-Wendisch et al., Microbiology 144:915-927 (1998)	A97276 Y09548
Pyc P458s	Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S	EP1108790	
SigC	Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C)	EP1108790	AX120368 AX120085
SigD	RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	EP1108790	AX120753 AX127144
SigE	Sigma Factor E EC 2.7.7.6 (extracytoplasmic function alternative sigma factor E)	EP1108790	AX127146 AX121325
SigH	Sigma Factor H EC 2.7.7.6 (sigma factor SigH)	EP1108790	AX127145 AX120939
SigM	Sigma Factor M EC 2.7.7.6 (sigma factor SigM)	EP1108790	AX123500 AX127153
Tal	Transaldolase EC 2.2.1.2 (transaldolase)	WO0104325	AX076272
ThyA	Thymidylate Synthase EC 2.1.1.45 (thymidylate synthase)	EP1108790	AX121026 AX127145
Tkt	Transketolase EC 2.2.1.1 (transktolase)	Ikeda et al., NCBI	AB023377
Tpi	Triose Phosphate Isomerase EC 5.3.1.1 (triose phosphate isomerase)	Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	X59403
zwa1	Cell Growth Factor 1 (growth factor 1)	EP1111062	AX133781
Zwf	Glucose 6-phosphate 1-Dehydrogenase EC 1.1.1.49 (glucose 6-phosphate 1-dehydrogenase)	EP1108790 WO0104325	AX127148 AX121827 AX076272
Zwf A213T	Glucose 6-phosphate 1-Dehydrogenase EC 1.1.1.49 (glucose 6-phosphate 1-dehydrogenase) amino acid exchange A213T	EP1108790	

Table 5

Target sites for integration of open reading frames, genes and alleles of methionine production

Gene name	Description of the coded enzyme or protein	Reference	Access Number
BrnE	Transporter of branched-chain amino acids (branched-chain amino acid transporter)	EP1096010	AX137709 AX137714
BrnF	Transporter of branched-chain amino acids (branched-chain amino acid transporter)	EP1096010	AX137709 AX137714
BrnQ	Carrier protein of branched-chain amino acids (branched-chain amino acid transport system carrier protein)	Tauch et al., Archives of Microbiology 169(4):303-12 (1998) WO0100805 EP1108790	M89931 AX066841 AX127150
ccpA1	Catabolite Control Protein (catabolite control protein A1)	WO0100844 EP1108790	AX065267 AX127147
ccpA2	Catabolite Control Protein (catabolite control protein A2)	WO0100844 EP1108790	AX065267 AX121594
citA	Sensor Kinase CitA (sensor kinase CitA)	EP1108790	AX120161
citB	Transcription Regulator CitB (transcription regulator CitB)	EP1108790	AX120163
citE	Citrate Lyase EC 4.1.3.6 (citrate lyase)	WO0100844 EP1108790	AX065421 AX127146
ddh	Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase)	Ishino et al., Nucleic Acids Research 15: 3917 (1987) EP1108790	S07384 AX127152
gluA	Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluB	Glutamate-binding Protein (glutamate-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluC	Glutamate Transport Permease (glutamate transport	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191

	system permease)		
gluD	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
luxR	Transcription Regulator LuxR (transcription regulator LuxR)	WO0100842 EP1108790	AX065953 AX123320
luxS	Histidine Kinase LuxS (histidine kinase LuxS)	EP1108790	AX123323 AX127145
lysR1	Transcription Regulator LysR1 (transcription regulator LysR1)	EP1108790	AX064673 AX127144
lysR2	Transcription Activator LysR2 (transcription regulator LysR2)	EP1108790	AX123312
lysR3	Transcription Regulator LysR3 (transcription regulator LysR3)	WO0100842 EP1108790	AX065957 AX127150
menE	O-Succinylbenzoic Acid CoA Ligase EC 6.2.1.26 (O-succinylbenzoate CoA ligase)	WO0100843 EP1108790	AX064599 AX064193 AX127144
metD	Transcription Regulator MetD (transcription regulator MetD)	EP1108790	AX123327 AX127153
metK	Methionine Adenosyl Transferase EC 2.5.1.6 (S-adenosylmethionine synthetase)	WO0100843 EP1108790	AX063959 AX127148
pck	Phosphoenol Pyruvate Carboxykinase (phosphoenol pyruvate carboxykinase)	WO0100844	AJ269506 AX065053
pgi	Glucose 6-Phosphate Isomerase EC 5.3.1.9 (glucose-6-phosphate isomerase)	EP1087015 EP1108790	AX136015 AX127146
poxB	Pyruvate Oxidase EC 1.2.3.3 (pyruvate oxidase)	WO0100844 EP1096013	AX064959 AX137665
zwa2	Cell Growth Factor 2 (growth factor 2)	EP1106693 EP1108790	AX113822 AX127146

A "copy of an open reading frame (ORF), gene or allele of threonine production" is to be understood as meaning all the open reading frames, genes or alleles of which enhancement/over-expression can have the effect of improving threonine production.

These include, inter alia, the following open reading frames, genes or alleles: accBC, accDA, cstA, cysD, cysE, cysH, cysI, cysN, cysQ, dps, eno, fda, gap, gap2, gdh, gnd, hom, hom^{FBR}, lysC, $lysC^{FBR}$, msiK, opcA, oxyR, ppc, ppc^{FBR}, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, thrB, thrC, thrE, zwa1, zwf and zwf A213T. These are summarized and explained in Table 6. These include, in particular, the $lysC^{FBR}$ alleles which code for a "feed back" resistant aspartate kinase (See Table 2) and the hom^{FBR} alleles which code for a "feed back" resistant homoserine dehydrogenase.

The second, optionally third or fourth copy of the open reading frame (ORF), gene or allele of threonine production in question can be integrated at in each case a second, optionally third or fourth site. The following open reading frames, genes or nucleotide sequences, inter alia, can be used for this: ccpA1, ccpA2, citA, citB, citE, ddh, gluA, gluB, gluC, gluD, glyA, ilvA, ilvBN, ilvC, ilvD, luxR, luxS, lysR1, lysR2, lysR3, mdh, menE, metA, metD, pck, poxB, sigB and zwa2. These are summarized and explained in Table 7.

The sites mentioned include, of course, not only the coding regions of the open reading frames or genes mentioned, but also the regions or nucleotide sequences lying upstream which are responsible for expression and regulation, such as, for example, ribosome binding sites, promoters, binding sites for regulatory proteins, binding sites for regulatory ribonucleic acids and attenuators. These regions in general lie in a range of 1-800, 1-600, 1-400, 1-200, 1-100 or 1-50

nucleotides upstream of the coding region. In the same way, regions lying downstream, such as, for example, transcription terminators, are also included. These regions in general lie in a range of 1-400, 1-200, 1-100, 1-50 or 1-25 nucleotides downstream of the coding region.

Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

Table 6

Open reading frames, genes and alleles of threonine production

Name	Description of the coded enzyme or protein	Reference	Access Number
accBC	Acyl-CoA Carboxylase EC 6.3.4.14 (acyl-CoA carboxylase)	Jäger et al. Archives of Microbiology 166:76-82 (1996) EP1108790 WO0100805	U35023 AX123524 AX066441
accDA	Acetyl-CoA Carboxylase EC 6.4.1.2 (acetyl-CoA carboxylase)	EP1055725 EP1108790 WO0100805	 AX121013 AX066443
cstA	Carbon Starvation Protein A (carbon starvation protein A)	EP1108790 WO0100804	AX120811 AX066109
cysD	Sulfate Adenylyltransferase sub-unit II EC 2.7.7.4 (sulfate adenylyltransferase small chain)	EP1108790	AX123177
cysE	Serine Acetyltransferase EC 2.3.1.30 (serine acetyltransferase)	EP1108790 WO0100843	AX122902 AX063961
cysH	3'-Phosphoadenyl Sulfate Reductase EC 1.8.99.4 (3'-phosphoadenosine 5'-phosphosulfate reductase)	EP1108790 WO0100842	AX123178 AX066001
cysK	Cysteine Synthase EC 4.2.99.8 (cysteine synthase)	EP1108790 WO0100843	AX122901 AX063963
cysN	Sulfate Adenylyltransferase sub-unit I EC 2.7.7.4 (sulfate adenylyltransferase)	EP1108790	AX123176 AX127152
cysQ	Transport protein CysQ (transporter cysQ)	EP1108790 WO0100805	AX127145 AX066423
dps	DNA Protection Protein (protection during starvation protein)	EP1108790	AX127153
eno	Enolase EC 4.2.1.11 (enolase)	EP1108790 WO0100844 EP1090998 Hermann et al., Electrophoresis 19:3217-3221 (1998)	AX127146 AX064945 AX136862
fda	Fructose Bisphosphate Aldolase EC 4.1.2.13 (fructose bisphosphate aldolase)	van der Osten et al., Molecular Microbiology 3:1625-1637 (1989)	X17313
gap	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12	EP1108790 WO0100844 Eikmanns et al.,	AX127148 AX064941 X59403

	(glyceraldehyde 3-phosphate dehydrogenase)	Journal of Bacteriology 174:6076-6086(1992)	
gap2	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase 2)	EP1108790 WO0100844	AX127146 AX064939
gdh	Glutamate Dehydrogenase EC 1.4.1.4 (glutamate dehydrogenase)	EP1108790 WO0100844 Boermann et al., Molecular Microbiology 6:317-326 (1992); Guyonvarch et al., NCBI	AX127150 AX063811 X59404 X72855
gnd	6-Phosphogluconate Dehydrogenase EC 1.1.1.44 (6-phosphogluconate dehydrogenase)	EP1108790 WO0100844	AX127147 AX121689 AX065125
hom	Homoserine Dehydrogenase EC 1.1.1.3 (homoserine dehydrogenase)	Peoples et al., Molecular Microbiology 2:63-72 (1988)	Y00546
hom^{FBR}	Homoserine Dehydrogenase feedback resistant (fbr) EC 1.1.1.3 (homoserine dehydrogenase fbr)	Reinscheid et al., Journal of Bacteriology 173:3228-30 (1991)	
lysC	Aspartate Kinase EC 2.7.2.4 (aspartate kinase)	EP1108790 WO0100844 Kalinowski et al., Molecular Microbiology 5:1197-204 (1991)	AX120365 AX063743 X57226
$lysC^{FBR}$	Aspartate Kinase feedback resistent (fbr) EC 2.7.2.4 (aspartate kinase fbr)	see Table 2	
msiK	Sugar Importer (multiple sugar import protein)	EP1108790	AX120892
opcA	Glucose 6-Phosphate Dehydrogenase (subunit of glucose 6-phosphate dehydrogenase)	WO0104325	AX076272
oxyR	Transcription Regulator (transcriptional regulator)	EP1108790	AX122198 AX127149
ppc^{FBR}	Phosphoenol Pyruvate Carboxylase feedback resistent EC 4.1.1.31 (phosphoenol pyruvate carboxylase feedback resistant)	EP0723011 WO0100852	
ppc	Phosphoenol Pyruvate Carboxylase EC 4.1.1.31 (phosphoenol pyruvate carboxylase)	EP1108790 O'Reagan et al., Gene 77(2):237-251(1989)	AX127148 AX123554 M25819

pgk	Phosphoglycerate Kinase EC 2.7.2.3 (phosphoglycerate kinase)	EP1108790 WO0100844 Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	AX121838 AX127148 AX064943 X59403
pknA	Protein Kinase A (protein kinase A)	EP1108790	AX120131 AX120085
pknB	Protein Kinase B (protein kinase B)	EP1108790	AX120130 AX120085
pknD	Protein Kinase D (protein kinase D)	EP1108790	AX127150 AX122469 AX122468
pknG	Protein Kinase G (protein kinase G)	EP1108790	AX127152 AX123109
ppsA	Phosphoenol Pyruvate Synthase EC 2.7.9.2 (phosphoenol pyruvate synthase)	EP1108790	AX127144 AX120700 AX122469
ptsH	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H)	EP1108790 WO0100844	AX122210 AX127149 AX069154
ptsI	Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I)	EP1108790	AX122206 AX127149
ptsM	Glukose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II)	Lee et al., FEMS Microbiology Letters 119 (1-2):137-145 (1994)	L18874
pyc	Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase)	WO9918228 Peters-Wendisch et al., Microbiology 144:915-927 (1998)	A97276 Y09548
pyc P458S	Pyruvate Carboxylase EC 6.4.1.1 (pyruvate carboxylase) amino acid exchange P458S	EP1108790	
sigC	Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C)	EP1108790	AX120368 AX120085
sigD	RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	EP1108790	AX120753 AX127144
sigE	Sigma Factor E EC 2.7.7.6 (extracytoplasmic function alternative sigma factor E)	EP1108790	AX127146 AX121325
sigH	Sigma Factor H EC 2.7.7.6 (sigma factor SigH)	EP1108790	AX127145 AX120939
sigM	Sigma Factor M	EP1108790	AX123500

	EC 2.7.7.6 (sigma factor SigM)		AX127153
tal	Transaldolase EC 2.2.1.2 (transaldolase)	WO0104325	AX076272
thrB	Homoserine Kinase EC 2.7.1.39 (homoserine kinase)	Peoples et al., Molecular Microbiology 2:63-72 (1988)	Y00546
thrC	Threonine Synthase EC 4.2.99.2 (threonine synthase)	Han et al., Molecular Microbiology 4:1693-1702 (1990)	X56037
thrE	Threonine Exporter (threonine export carrier)	EP1085091	AX137526
thyA	Thymidylate Synthase EC 2.1.1.45 (thymidylate synthase)	EP1108790	AX121026 AX127145
tkt	Transketolase EC 2.2.1.1 (transketolase)	Ikeda et al., NCBI	AB023377
tpi	Triose phosphate Isomerase EC 5.3.1.1 (triose phosphate isomerase)	Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	X59403
zwa1	Cell Growth Factor 1 (growth factor 1)	EP1111062	AX133781
zwf	Glucose 6-Phosphate 1-Dehydrogenase EC 1.1.1.49 (glucose 6-phosphate 1-dehydrogenase)	EP1108790 WO0104325	AX127148 AX121827 AX076272
zwf A213T	Glucose 6-Phosphate 1-Dehydrogenase EC 1.1.1.49 (glucose 6-phosphate 1-dehydrogenase) amino acid exchange A213T	EP1108790	

Table 7

Target sites for integration of open reading frames, genes and alleles of threonine production

Gene name	Description of the coded enzyme or protein	Reference	Access Number
ccpA1	Catabolite Control Protein (catabolite control protein A1)	WO0100844 EP1108790	AX065267 AX127147
ccpA2	Catabolite Control Protein (catabolite control protein A2)	WO0100844 EP1108790	AX065267 AX121594
citA	Sensor Kinase CitA (sensor kinase CitA)	EP1108790	AX120161
citB	Transcription Regulator CitB (transcription regulator CitB)	EP1108790	AX120163
citE	Citrate Lyase EC 4.1.3.6 (citrate lyase)	WO0100844 EP1108790	AX065421 AX127146
ddh	Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase)	Ishino et al., Nucleic Acids Research 15: 3917 (1987) EP1108790	S07384 AX127152
gluA	Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluB	Glutamate-binding Protein (glutamate-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluC	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluD	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
glyA	Glycine Hydroxymethyltransferase EC 2.1.2.1 (glycine hydroxymethyltransferase)	WO0100843	AX063861 AF327063
ilvA	Threonine Dehydratase EC 4.2.1.16 (threonine dehydratase)	Möckel et al., Journal of Bacteriology 174 (24), 8065-8072 (1992) EP1108790	A47044 L01508 AX127150
ilvBN	Acetolactate Synthase EC 4.1.3.18	Keilhauer et al., Journal of Bacteriology	L09232

	(acetolactate synthase)	175(17):5595-603 (1993) EP1108790	AX127147
ilvC	Reductoisomerase EC 1.1.1.86 (ketol-acid reductoisomerase)	Keilhauer et al., Journal of Bacteriology 175(17):5595-603 (1993) EP1108790	C48648 AX127147
ilvD	Dihydroxy-acid Dehydratase EC 4.2.1.9 (dihydroxy-acid dehydratase)	EP1006189	AX136925
luxR	Transcription Regulator LuxR (transcription regulator LuxR)	WO0100842 EP1108790	AX065953 AX123320
luxS	Histidine Kinase LuxS (histidine kinase LuxS)	EP1108790	AX123323 AX127153
lysR1	Transcription Regulator LysR1 (transcription regulator LysR1)	EP1108790	AX064673 AX127144
lysR2	Transcription Activator LysR2 (transcription regulator LysR2)	EP1108790	AX123312
lysR3	Transcription Regulator LysR3 (transcription regulator LysR3)	WO0100842 EP1108790	AX065957 AX127150
mdh	Malate Dehydrogenase EC 1.1.1.37 (malate dehydrogenase)	WO0100844	AX064895
menE	O-Succinylbenzoic Acid CoA Ligase EC 6.2.1.26 (O-succinylbenzoate CoA ligase)	WO0100843 EP1108790	AX064599 AX064193 AX127144
metA	Homoserine O-Acetyltransferase EC 2.3.1.31 (homoserine O-acetyltransferase)	Park et al., Molecular Cells 30;8(3):286-94 (1998) WO0100843 EP1108790	AX063895 AX127145
metD	Transcription Regulator MetD (transcription regulator MetD)	EP1108790	AX123327 AX127153
pck	Phosphoenol Pyruvate Carboxykinase (phosphoenol pyruvate carboxykinase)	WO0100844	AJ269506 AX065053
poxB	Pyruvate Oxidase EC 1.2.3.3 (pyruvate oxidase)	WO0100844 EP1096013	AX064959 AX137665
sigB	RNA Polymerase Transcription Factor (RNA polymerase transcription factor)	EP1108790	AX127149

zwa2	Cell Growth Factor 2 (growth factor 2)	EP1106693 EP1108790	AX113822 AX127146

The invention accordingly also provides a process for the production of coryneform bacteria which produce L-methionine and/or L-threonine, which comprises

a) isolating the nucleotide sequence of at least one desired ORF, gene or allele of methionine production or threonine production, optionally including the expression and/or regulation signals,

b) providing the 5' and the 3' end of the ORF, gene or allele with nucleotide sequences of the target site,

c) preferably incorporating the nucleotide sequence of the desired ORF, gene or allele provided with nucleotide sequences of the target site into a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,

d) transferring the nucleotide sequence according to b) or c) into coryneform bacteria, and

e) isolating coryneform bacteria in which the nucleotide sequence according to a) is incorporated at the target site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the target site.

The invention furthermore provides coryneform bacteria, in particular of the genus Corynebacterium, which produce L-valine, wherein these have, in addition to at least one of the copy of an open reading frame (ORF), gene or allele of valine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the

open reading frame (ORF), gene or allele in question at in each case a second, optionally third or fourth site in integrated form, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular second, optionally third or fourth site.

The invention also furthermore provides a process for the preparation of L-valine, which comprises the following steps:

a) fermentation of coryneform bacteria, in particular Corynebacterium glutamicum, characterized in that these have, in addition to at least one of the copy of an open reading frame (ORF), gene or allele of valine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at in each case a second, optionally third or fourth site in integrated form, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics being present at the particular second, optionally third or fourth site,

under conditions which allow expression of the said open reading frames (ORF), genes or alleles,

b) concentration of the L-valine in the fermentation broth,

c) isolation of the L-valine from the fermentation broth, optionally

d) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100%.

A "copy of an open reading frame (ORF), gene or allele of valine production" is to be understood as meaning all the open reading frames, genes or alleles of which enhancement/over-expression can have the effect of improving valine production.

These include, inter alia, the following open reading frames, genes or alleles: brnE, brnF, brnEF, cstA, cysD, dps, eno, fda, gap, gap2, gdh, ilvB, ilvN, ilvBN, ilvC, ilvD, ilvE msiK, pgk, ptsH, ptsI, ptsM, sigC, sigD, sigE, sigH, sigM, tpi, zwa1. These are summarized and explained in Table 8. These include in particular the acetolactate synthase which codes for a valine-resistant.

The second, optionally third or fourth copy of the open reading frame (ORF), gene or allele of threonine production in question can be integrated at in each case a second, optionally third or fourth site. The following open reading frames, genes or nucleotide sequences, inter alia, can be used for this: aecD, ccpA1, ccpA2, citA, citB, citE, ddh, gluA, gluB, gluC, gluD, glyA, ilvA, luxR, lysR1, lysR2, lysR3, panB, panC, poxB and zwa2. These are summarized and explained in Table 9.

The sites mentioned include, of course, not only the coding regions of the open reading frames or genes mentioned, but also the regions or nucleotide sequences lying upstream which are responsible for expression and regulation, such as, for example, ribosome binding sites, promoters, binding sites for regulatory proteins, binding sites for regulatory ribonucleic acids and attenuators. These regions in general lie in a range of 1-800, 1-600, 1-400, 1-200, 1-100 or 1-50 nucleotides upstream of the coding region. In the same way, regions lying downstream, such as, for example,

transcription terminators, are also included. These regions in general lie in a range of 1-400, 1-200, 1-100, 1-50 or 1-25 nucleotides downstream of the coding region.

Intergenic regions in the chromosome, that is to say nucleotide sequences without a coding function, can furthermore be used. Finally, prophages or defective phages contained in the chromosome can be used for this.

Table 8

Open reading frames, genes and alleles of valine production

Name	Description of the coded enzyme or protein	Reference	Access Number
brnEF	Export of branched-chain amino acids (branched chain amino acid export)	EP1096010 Kennerknecht et al., NCBI	AF454053
cstA	Carbon Starvation Protein A (carbon starvation protein A)	EP1108790 WO0100804	AX120811 AX066109
dps	DNA Protection Protein (protection during starvation protein)	EP1108790	AX127153
eno	Enolase EC 4.2.1.11 (enolase)	EP1108790 WO0100844 EP1090998 Hermann et al., Electrophoresis 19:3217-3221 (1998)	AX127146 AX064945 AX136862
fda	Fructose Bisphosphate Aldolase EC 4.1.2.13 (fructose bisphosphate aldolase)	van der Osten et al., Molecular Microbiology 3:1625-1637 (1989)	X17313
gap	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase)	EP1108790 WO0100844 Eikmanns et al., Journal of Bacteriology 174:6076-6086(1992)	AX127148 AX064941 X59403
gap2	Glyceraldehyde 3-Phosphate Dehydrogenase EC 1.2.1.12 (glyceraldehyde 3-phosphate dehydrogenase 2)	EP1108790 WO0100844	AX127146 AX064939
gdh	Glutamate Dehydrogenase EC 1.4.1.4 (glutamate dehydrogenase)	EP1108790 WO0100844 Boermann et al., Molecular Microbiology 6:317-326 (1992); Guyonvarch et al., NCBI	AX127150 AX063811 X59404 X72855
ilvBN	Acetolactate Synthase EC 4.1.3.18 (acetolactate synthase)	Keilhauer et al., Journal of Bacteriology 175(17):5595-603 (1993) EP1108790	L09232 AX127147
ilvC	Isomeroreductase EC 1.1.1.86	Keilhauer et al., Journal of	C48648 AX127147

	(acetohydroxy acid isomeroreductase)	Bacteriology 175(17):5595-603 (1993) EP1108790	
ilvD	Dihydroxy-acid Dehydratase EC 4.2.1.9 (dihydroxy acid dehydratase)	EP1006189	AX136925
ilvE	Transaminase B EC 2.6.1.42 (transaminase B)	EP1108790	AX127150 AX122498
msiK	Sugar Importer (multiple sugar import protein)	EP1108790	AX120892
pgk	Phosphoglycerate Kinase EC 2.7.2.3 (phosphoglycerate kinase)	EP1108790 WO0100844 Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	AX127148 AX064943 X59403
ptsH	Phosphotransferase System Protein H EC 2.7.1.69 (phosphotransferase system component H)	EP1108790 WO0100844	AX122210 AX127149 AX069154
ptsI	Phosphotransferase System Enzyme I EC 2.7.3.9 (phosphotransferase system enzyme I)	EP1108790	AX122206 AX127149
ptsM	Glucose-specific Phosphotransferase System Enzyme II EC 2.7.1.69 (glucose phosphotransferase-system enzyme II)	Lee et al., FEMS Microbiology Letters 119 (1-2):137-145 (1994)	L18874
sigC	Sigma Factor C EC 2.7.7.6 (extracytoplasmic function alternative sigma factor C)	EP1108790	AX120368 AX120085
sigD	RNA Polymerase Sigma Factor D EC 2.7.7.6 (RNA polymerase sigma factor)	EP1108790	AX120753 AX127144
sigE	Sigma Factor E EC 2.7.7.6 (extracytoplasmic function alternative sigma factor E)	EP1108790	AX127146 AX121325
sigH	Sigma Factor H EC 2.7.7.6 (sigma factor SigH)	EP1108790	AX127145 AX120939
sigM	Sigma Factor M EC 2.7.7.6 (sigma factor SigM)	EP1108790	AX123500 AX127153
tpi	Triose Phosphate Isomerase EC 5.3.1.1 (triose phosphate isomerase)	Eikmanns, Journal of Bacteriology 174:6076-6086 (1992)	X59403
zwa1	Cell Growth Factor 1 (growth factor 1)	EP1111062	AX133781

Table 9

Target sites for integration of open reading frames, genes and alleles of valine production

Gene name	Description of the coded enzyme or protein	Reference	Access Number
aecD	beta C-S Lyase EC 2.6.1.1 (beta C-S lyase)	Rossol et al., Journal of Bacteriology 174(9):2968-77 (1992)	M89931
ccpA1	Catabolite Control Protein (catabolite control protein A1)	WO0100844 EP1108790	AX065267 AX127147
ccpA2	Catabolite Control Protein (catabolite control protein A2)	WO0100844 EP1108790	AX065267 AX121594
citA	Sensor Kinase CitA (sensor kinase CitA)	EP1108790	AX120161
citB	Transcription Regulator CitB (transcription regulator CitB)	EP1108790	AX120163
citE	Citrate Lyase EC 4.1.3.6 (citrate lyase)	WO0100844 EP1108790	AX065421 AX127146
ddh	Diaminopimelate Dehydrogenase EC 1.4.1.16 (diaminopimelate dehydrogenase)	Ishino et al., Nucleic Acids Research 15: 3917 (1987) EP1108790	S07384 AX127152
gluA	Glutamate Transport ATP-binding Protein (glutamate transport ATP-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluB	Glutamate-binding Protein (glutamate-binding protein)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluC	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
gluD	Glutamate Transport Permease (glutamate transport system permease)	Kronemeyer et al., Journal of Bacteriology 177(5):1152-8 (1995)	X81191
glyA	Glycine Hydroxymethyltransferase EC 2.1.2.1 (glycine hydroxymethyltransferase)	WO0100843	AX063861 AF327063
ilvA	Threonine Dehydratase EC 4.2.1.16 (threonine dehydratase)	Möckel et al., Journal of Bacteriology 174 (24), 8065-8072 (1992)	A47044 L01508 AX127150

		EP1108790	
luxR	Transcription Regulator LuxR (transcription regulator LuxR)	WO0100842 EP1108790	AX065953 AX123320
lysR1	Transcription Regulator LysR1 (transcription regulator LysR1)	EP1108790	AX064673 AX127144
lysR2	Transcription Activator LysR2 (transcription regulator LysR2)	EP1108790	AX123312
lysR3	Transcription Regulator LysR3 (transcription regulator LysR3)	WO0100842 EP1108790	AX065957 AX127150
panB	Ketopantoate Hydroxymethyltransferase EC 2. 1. 2. 11 (ketopantoate hydroxymethyltransferase)	US6177264	X96580
panC	Pantothenate Synthetase EC 6.3.2.1 (pantothenate synthetase)	US6177264	X96580
poxB	Pyruvate Oxidase EC 1.2.3.3 (pyruvate oxidase)	WO0100844 EP1096013	AX064959 AX137665
zwa2	Cell Growth Factor 2 (growth factor 2)	EP1106693 EP1108790	AX113822 AX127146

The invention accordingly also provides a process for the production of coryneform bacteria which produce L-valine, which comprises

a) isolating the nucleotide sequence of at least one desired ORF, gene or allele of valine production, optionally including the expression and/or regulation signals,

b) providing the 5' and the 3' end of the ORF, gene or allele with nucleotide sequences of the target site,

c) preferably incorporating the nucleotide sequence of the desired ORF, gene or allele provided with nucleotide sequences of the target site into a vector

which does not replicate or replicates to only a limited extent in coryneform bacteria,

d) transferring the nucleotide sequence according to b) or c) into coryneform bacteria, and

e) isolating coryneform bacteria in which the nucleotide sequence according to a) is incorporated at the target site, no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remaining at the target site.

During work on the present invention, it was possible to incorporate a second copy of an $lysC^{FBR}$ allele into the gluB gene of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the gluB gene site. This strain, which is called DSM13994glu::lysC, carries the $lysC^{FBR}$ allele lysC T311I at its natural lysC site and a second copy of the $lysC^{FBR}$ allele lysC T311I at a second site (target site), namely the gluB gene. A plasmid with the aid of which the incorporation of the $lysC^{FBR}$ allele into the gluB gene can be achieved is shown in Figure 1. It carries the name pK18mobsacBglu1_1.

During work on the present invention, it was furthermore possible to incorporate a copy of an $lysC^{FBR}$ allele into the target site of the gluB gene of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the gluB gene site.

This strain, which is called DSM12866glu::lysC, carries the wild-type form of the lysC gene at its natural lysC site and a second copy of the lysC gene in the form of the $lysC^{FBR}$ allele lysC T311I at a second site (target site), namely the gluB gene. It has been deposited under number DSM15039 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures). A plasmid with the aid of which the incorporation of the $lysC^{FBR}$ allele into the gluB gene can be achieved is shown in Figure 1. It carries the name pK18mobsacBglu1_1.

During work on the present invention, it was furthermore possible to incorporate a copy of an $lysC^{FBR}$ allele into the target site of the aecD gene of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the aecD gene site. This strain, which is called DSM12866aecD::lysC, carries the wild-type form of the lysC gene at its natural lysC site and a second copy of the lysC gene in the form of the $lysC^{FBR}$ allele lysC T311I at a second site (target site), namely the aecD gene. A plasmid with the aid of which the incorporation of the $lysC^{FBR}$ allele into the aecD gene can be achieved is shown in Figure 2. It carries the name pK18mobsacBaecD1_1.

During work on the present invention, it was furthermore possible to incorporate a copy of an $lysC^{FBR}$ allele into the target site of the pck gene of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the pck gene site.

This strain, which is called DSM12866pck::lysC, carries the wild-type form of the lysC gene at its natural lysC site and a second copy of the lysC gene in the form of the $lysC^{FBR}$ allele lysC T311I at a second site (target site), namely the pck gene. A plasmid with the aid of which the incorporation into the pck gene can be achieved is shown in Figure 3. It carries the name pK18mobsacBpck1_1.

During work on the present invention, it was furthermore possible to incorporate a copy of the ddh gene into the target site of the gluB gene of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the gluB gene site. This strain, which is called DSM12866glu::ddh, carries a copy of the ddh gene at its natural ddh site and a second copy of the ddh gene at a second site (target site), namely the gluB gene. A plasmid with the aid of which the incorporation of the ddh gene into the gluB gene can be achieved is shown in Figure 4. It carries the name pK18mobsacBgluB2_1.

During work on the present invention, it was furthermore possible to incorporate a copy of the dapA gene into the target site of the aecD gene of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the aecD gene site. This strain, which is called DSM12866aecD::dapA, carries a copy of the dapA gene at its natural dapA site and a second copy of the dapA gene at a second site (target site), namely the aecD gene. A plasmid with the aid of which the incorporation of the dapA gene into the aecD gene can be

achieved is shown in Figure 5. It carries the name pK18mobsacBaecD2_1.

During work on the present invention, it was furthermore possible to incorporate a copy of a pyc allele into the target site of the pck gene of Corynebacterium glutamicum such that no nucleotide sequence which is capable of/enables episomal replication in microorganisms, no nucleotide sequence which is capable of/enables transposition and no nucleotide sequence which imparts resistance to antibiotics remained at the pck gene site. This strain, which is called DSM12866pck::pyc, carries a copy of the wild-type form of the pyc gene at its natural pyc site and a second copy of the pyc gene in the form of the pyc allele pyc P458S at a second site (target site), namely the pck gene. A plasmid with the aid of which the incorporation of the pyc allele into the pck gene can be achieved is shown in Figure 6. It carries the name pK18mobsacBpck1_3.

The coryneform bacteria produced according to the invention can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of chemical compounds. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid or lactic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e. g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as e.g.

antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20ºC to 45ºC, and preferably 25ºC to 40ºC. Culturing is continued until a maximum of the desired chemical compound has formed. This target is usually reached within 10 hours to 160 hours.

It has been found that the coryneform bacteria according to the invention, in particular the coryneform bacteria which produce L-lysine, have an unexpectedly high stability. They were stable for at least 10-20, 20-30, 30-40, 40-50, preferably at least 50-60, 60-70, 70-80 and 80-90 generations or cell division cycles.

The following microorganisms have been deposited:

The strain Corynebacterium glutamicum DSM12866glu::lysC was deposited in the form of a pure culture on 5th June 2002 under number DSM15039 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

The plasmid pK18mobsacBglu1_1 was deposited in the form of a pure culture of the strain E. coli DH5αmcr/pK18mobsacBglu1_1 (= DH5alphamcr/pK18mobsacBglu1_1) on 20th April 2001 under number DSM14243 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

The plasmid pK18mobsacBaecD1_1 was deposited in the form of a pure culture of the strain E. coli DH5αmcr/pK18mobsacBaecD1_1 (= DH5alphamcr/pK18mobsacBaecD1_1) on 5th June 2002 under number DSM15040 at the Deutsche Sammlung für

Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

Example 1

Incorporation of a second copy of the $lysC^{FBR}$ allele into the chromosome of the strain DSM13994 and of the strain DSM12866

The Corynebacterium glutamicum strain DSM13994 was produced by multiple, non-directed mutagenesis, selection and mutant selection from C. glutamicum ATCC13032. The strain is resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine and has a feed back-resistant aspartate kinase which is insensitive to inhibition by a mixture of lysine and threonine (in each case 25 mM). The nucleotide sequence of the $lysC^{FBR}$ allele of this strain is shown as SEQ ID NO:3. It is also called lysC T311I in the following. The amino acid sequence of the aspartate kinase protein coded is shown as SEQ ID NO:4. A pure culture of this strain was deposited on 16th January 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

The strain DSM12866 was produced from C. glutamicum ATCC13032 by non-directed mutagenesis and selection of the mutants with the best L-lysine accumulation. It is methionine-sensitive. Growth on minimal medium comprising L-methionine can be re-established by addition of threonine. This strain has the wild-type form of the lysC gene shown as SEQ ID NO:1. The corresponding amino acid sequence of the wild-type aspartate kinase protein is shown as SEQ ID NO:2. A pure culture of this strain was deposited on 10th June 1999 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

1.1 Isolation and sequencing of the DNA of the lysC allele of strain DSM13994

From the strain DSM13994, chromosomal DNA is isolated by the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). With the aid of the polymerase chain reaction, a DNA section which carries the lysC gene or allele is amplified. On the basis of the sequence of the lysC gene known for C. glutamicum (Kalinowski et al., Molecular Microbiology, 5 (5), 1197 - 1204 (1991); Accession Number X57226), the following primer oligonucleotides were chosen for the PCR:

lysC1beg (SEQ ID No: 5):
5` TA(G GAT CC)T CCG GTG TCT GAC CAC GGT G 3`

lysC2end: (SEQ ID NO: 6):
5` AC(G GAT CC)G CTG GGA AAT TGC GCT CTT CC 3`

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA section of approx. 1.7 kb in length, which carries the lysC gene or allele. The primers moreover contain the sequence for a cleavage site of the restriction endonuclease BamHI, which is marked by parentheses in the nucleotide sequence shown above.

The amplified DNA fragment of approx. 1.7 kb in length which carries the lysC allele of the strain DSM13994 is identified by electrophoresis in a 0.8% agarose gel, isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

Ligation of the fragment is then carried out by means of the Topo TA Cloning Kit (Invitrogen, Leek, The Netherlands, Cat. Number K4600-01) in the vector pCRII-TOPO. The ligation batch is transformed in the E. coli strain TOP10

(Invitrogen, Leek, The Netherlands). Selection of plasmid-carrying cells is made by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, 64 mg/l).

The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The resulting plasmid is called pCRIITOPOlysC.

The nucleotide sequence of the amplified DNA fragment or PCR product is determined by the dideoxy chain termination method of Sanger et al. (Proceedings of the National Academy of Sciences USA, 74:5463-5467 (1977)) using the "ABI Prism 377" sequencing apparatus of PE Applied Biosystems (Weiterstadt, Germany). The sequence of the coding region of the PCR product is shown in SEQ ID No:3. The amino acid sequence of the associated aspartate kinase protein is shown in SEQ ID NO:4.

The base thymine is found at position 932 of the nucleotide sequence of the coding region of the $lysC^{FBR}$ allele of strain DSM13994 (SEQ ID NO:3). The base cytosine is found at the corresponding position of the wild-type gene (SEQ ID NO:1).

The amino acid isoleucine is found at position 311 of the amino acid sequence of the aspartate kinase protein of strain DSM13994 (SEQ ID No:4). The amino acid threonine is found at the corresponding position of the wild-type protein (SEQ ID No:2).

The lysC allele, which contains the base thymine at position 932 of the coding region and accordingly codes for an aspartate kinase protein which contains the amino acid isoleucine at position 311 of the amino acid sequence, is called the $lysC^{FBR}$ allele or lysC T311I in the following.

The plasmid pCRIITOPOlysC, which carries the $lysC^{FBR}$ allele lysC T311I, was deposited in the form of a pure culture of the strain E. coli TOP 10/pCRIITOPOlysC under number DSM14242 on 20th April 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

1.2 Construction of the replacement vector pK18mobsacBglu1_1

The Corynebacterium glutamicum strain ATCC13032 is used as the donor for the chromosomal DNA. From the strain ATCC13032, chromosomal DNA is isolated using the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). With the aid of the polymerase chain reaction, a DNA fragment which carries the gluB gene and surrounding regions is amplified. On the basis of the sequence of the gluABCD gene cluster known for C. glutamicum (Kronemeyer et al., Journal of Bacteriology, 177: 1152 - 1158 (1995)) (Accession Number X81191), the following primer oligonucleotides are chosen for the PCR:

gluBgl1 (SEQ ID NO: 7):
5` TA(A GAT CT)G TGT TGG ACG TCA TGG CAA G 3`

gluBgl2 (SEQ ID NO: 8):
5` AC(A GAT CT)T GAA GCC AAG TAC GGC CAA G 3`

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx 1.7 kb in size, which carries the gluB gene and surrounding regions. The surrounding regions are a sequence section approx. 0.33 kb in length upstream of the gluB gene, which represents the 3' end of the gluA gene, and a sequence section approx. 0.44 kb in length downstream of the gluB gene, which

represents the 5' end of the gluC gene. The primers moreover contain the sequence for the cleavage site of the restriction endonuclease BglII, which is marked by parentheses in the nucleotide sequence shown above.

The amplified DNA fragment of approx. 1.7 kb in length which carries the gluB gene and surrounding regions is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

Ligation of the fragment is then carried out by means of the TOPO TA Cloning Kit (Invitrogen, Leek, The Netherlands, Cat. Number K4600-01) in the vector pCRII-TOPO. The ligation batch is transformed in the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands). Selection of plasmid-carrying cells is made by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, 64 mg/l).

The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The resulting plasmid is called pCRII-TOPOglu.

The plasmid pCRII-TOPOglu is cleaved with the restriction enzyme BglII (Amersham-Pharmacia, Freiburg, Germany) and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the gluB fragment of approx. 1.7 kb is isolated from the agarose gel and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al. (Gene 14: 69-73 (1994)). This is cleaved beforehand with the restriction enzyme BamHI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the gluB fragment of approx. 1.7 kb, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBglu1.

Plasmid DNA was isolated from the strain DSM14242 (see Example 1.1), which carries the plasmid pCRIITOPOlysC, and cleaved with the restriction enzyme BamHI (Amersham-Pharmacia, Freiburg, Germany), and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the $lysC^{FBR}$-containing DNA fragment of approx. 1.7 kb in length was isolated from the agarose gel and employed for ligation with the vector pK18mobsacBglu1 described above. This is cleaved beforehand with the restriction enzyme BamHI, dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim, Germany), mixed with the $lysC^{FBR}$ fragment of approx. 1.7 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5αmcr (Life Technologies GmbH, Karlsruhe, Germany) is then transformed with the ligation batch (Hanahan, In: DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold

Spring Harbor, New York, 1989), which was supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBglu1_1. A map of the plasmid is shown in Figure 1.

The plasmid pK18mobsacBglu1_1 was deposited in the form of a pure culture of the strain E. coli DH5αmcr/pK18mobsacBglu1_1 (= DH5alphamcr/pK18mobsacBglu1_1) under number DSM14243 on 20.04.2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

1.3 Incorporation of a second copy of the $lysC^{FBR}$ allele lysC T311I into the chromosome (target site: gluB gene) of the strain DSM13994 by means of the replacement vector pK18mobsacBglu1_1

The vector pK18mobsacBglu1_1 described in Example 1.2 is transferred by the protocol of Schäfer et al. (Journal of Microbiology 172: 1663-1666 (1990)) into the C. glutamicum strain DSM13994 by conjugation. The vector cannot replicate independently in DSM13994 and is retained in the cell only if it has integrated into the chromosome. Selection of clones or transconjugants with integrated pK18mobsacBglu1_1 is made by plating out the conjugation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 15 mg/l kanamycin and 50 mg/l nalidixic acid. Kanamycin-resistant transconjugants are plated out on LB agar plates with 25 mg/l kanamycin and incubated for 48 hours at 33°C.

For selection of mutants in which excision of the plasmid has taken place as a consequence of a second recombination event, the clones are cultured for 20 hours in LB liquid medium and then plated out on LB agar with 10% sucrose and incubated for 48 hours.

The plasmid pK18mobsacBglu1_1, like the starting plasmid pK18mobsacB, contains, in addition to the kanamycin resistance gene, a copy of the sacB gene which codes for levan sucrase from Bacillus subtilis. The expression which can be induced by sucrose leads to the formation of levan sucrase, which catalyses the synthesis of the product levan, which is toxic to C. glutamicum. Only those clones in which the integrated pK18mobsacBglu1_1 has excised as the consequence of a second recombination event therefore grow on LB agar. Depending on the position of the second recombination event, after the excision the second copy of the $lysC^{FBR}$ allele manifests itself in the chromosome at the gluB locus, or the original gluB locus of the host remains.

Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". Approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin" are investigated with the aid of the polymerase chain reaction. A DNA fragment which carries the gluB gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The same primer oligonucleotides as are described in Example 1.2 for the construction of the integration plasmid are chosen for the PCR.

gluBgl1 (SEQ ID NO: 7):
5` TA(A GAT CT)G TGT TGG ACG TCA TGG CAA G 3`

gluBgl2 (SEQ ID NO: 8):
5` AC(A GAT CT)T GAA GCC AAG TAC GGC CAA G 3`

The primers allow amplification of a DNA fragment approx. 1.7 kb in size in control clones with the original gluB locus. In clones with a second copy of the $lysC^{FBR}$ allele in the chromosome at the gluB locus, DNA fragments with a size of approx. 3.4 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel.

A clone which, in addition to the copy present at the lysC locus, has a second copy of the $lysC^{FRB}$ allele lysC T311I at the gluB locus in the chromosome was identified in this manner. This clone was called strain DSM13994glu::lysC.

1.4 Incorporation of a second copy of the lysC gene in the form of the $lysC^{FBR}$ allele lysC T311I into the chromosome (target site: gluB gene) of the strain DSM12866 by means of the replacement vector pK18mobsacBglu1_1

As described in Example 1.3, the plasmid pK18mobsacBglu1_1 is transferred into the C. glutamicum strain DSM12866 by conjugation. A clone which, in addition to the copy of the wild-type gene present at the lysC locus, has a second copy of the lysC gene in the form of the $lysC^{FBR}$ allele lysC T311I at the gluB locus in the chromosome was identified in the manner described in 1.3. This clone was called strain DSM12866glu::lysC.

The Corynebacterium glutamicum strain according to the invention which carries a second copy of an $lysC^{FBR}$ allele in the gluB gene was deposited in the form of a pure culture of the strain Corynebacterium glutamicum DSM12866glu::lysC on 5th June 2002 under number DSM15039 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

1.5 'Construction of the replacement vector pK18mobsacBpck1_1

The Corynebacterium glutamicum strain ATCC13032 is used as the donor for the chromosomal DNA. From the strain ATCC13032, chromosomal DNA is isolated using the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). With the aid of the polymerase chain reaction, a DNA fragment which carries the pck gene and surrounding regions is amplified. On the basis of the sequence of the pck gene known for C. glutamicum (EP1094111 and Riedel et al., Journal of Molecular and Microbiological Biotechnology 3:573-583 (2001)) (Accession Number AJ269506), the following primer oligonucleotides are chosen for the PCR:

pck_beg (SEQ ID NO: 9):
5` TA(A GAT CT) G CCG GCA TGA CTT CAG TTT 3`

pck_end (SEQ ID NO: 10):
5` AC(A GAT CT) G GTG GGA GCC TTT CTT GTT ATT3`

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx. 2.9 kb in size, which carries the pck gene and adjacent regions. The primers moreover contain the sequence for the cleavage site of the restriction endonuclease BglII, which is marked by parentheses in the nucleotide sequence shown above.

The amplified DNA fragment of approx. 2.9 kb in length which carries the pck gene and surrounding regions is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

Ligation of the fragment is then carried out by means of the TOPO TA Cloning Kit (Invitrogen, Leek, The Netherlands, Cat. Number K4600-01) in the vector pCRII-TOPO. The ligation batch is transformed in the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands). Selection of plasmid-carrying cells is made by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal (64 mg/l).

The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The resulting plasmid is called pCRII-TOPOpck.

The plasmid pCRII-TOPOpck is cleaved with the restriction enzyme BglII (Amersham-Pharmacia, Freiburg, Germany) and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the pck fragment of approx. 2.9 kb is isolated from the agarose gel and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al. (Gene 14: 69-73 (1994)). This is cleaved beforehand with the restriction enzyme BamHI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the pck fragment of approx. 2.9 kb, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli Strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989) Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBpck1.

Plasmid DNA was isolated from the strain DSM14242 (see Example 1.1), which carries the plasmid pCRIITOPOlysC, and cleaved with the restriction enzyme BamHI (Amersham-Pharmacia, Freiburg, Germany), and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the $lysC^{FBR}$-containing DNA fragment approx. 1.7 kb long was isolated from the agarose gel and employed for ligation with the vector pK18mobsacBpck1 described above. This is cleaved beforehand with the restriction enzyme BamHI, dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim, Germany), mixed with the $lysC^{FBR}$ fragment of approx. 1.7 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5αmcr (Life Technologies GmbH, Karlsruhe, Germany) is then transformed with the ligation batch (Hanahan, In: DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobdsacBpck1_1. A map of the plasmid is shown in Figure 3.

1.6 Incorporation of a second copy of the lysC gene in the form of the $lysC^{FBR}$ allele lysC T311I into the chromosome (target site: pck gene) of the strain DSM12866 by means of the replacement vector pK18mobsacBpck1_1

As described in Example 1.3, the plasmid pK18mobsacBpck1_1 described in Example 1.5 is transferred into the C. glutamicum strain DSM12866 by conjugation. Selection is made for targeted recombination events in the chromosome of C. glutamicum DSM12866 as described in Example 1.3. Depending on the position of the second recombination event, after the excision the second copy of the $lysC^{FBR}$ allele manifests itself in the chromosome at the pck locus, or the original pck locus of the host remains.

Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". Approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin" are investigated with the aid of the polymerase chain reaction. A DNA fragment which carries the pck gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The same primer oligonucleotides as are described in Example 1.5 for the construction of the integration plasmid are chosen for the PCR.

pck_beg (SEQ ID NO: 9):
5` TA(A GAT CT) G CCG GCA TGA CTT CAG TTT 3`

pck_end (SEQ ID NO: 10):
5` AC(A GAT CT) G GTG GGA GCC TTT CTT GTT ATT3`

The primers allow amplification of a DNA fragment approx. 2.9 kb in size in control clones with the original pck locus. In clones with a second copy of the $lysC^{FBR}$ allele in

the chromosome at the pck locus, DNA fragments with a size of approx. 4.6 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel.

A clone which, in addition to the copy of the wild-type gene present at the lysC locus, has a second copy of the lysC gene in the form of the $lysC^{FBR}$ allele lysC T311I at the pck locus in the chromosome was identified in this manner. This clone was called strain DSM12866pck::lysC.

1.7 Construction of the replacement vector pK18mobsacBaecD1_1

The Corynebacterium glutamicum strain ATCC13032 is used as the donor for the chromosomal DNA. From the strain ATCC13032, chromosomal DNA is isolated using the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). With the aid of the polymerase chain reaction, a DNA fragment which carries the aecD gene and surrounding regions is amplified. On the basis of the sequence of the aecD gene known for C. glutamicum (Rossol et al., Journal of Bacteriology 174:2968-2977 (1992)) (Accession Number M89931), the following primer oligonucleotides are chosen for the PCR:

aecD_beg (SEQ ID NO: 11):
5` GAA CTT ACG CCA AGC TGT TC 3`

aecD_end (SEQ ID NO: 12):
5` AGC ACC ACA ATC AAC GTG AG 3`

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx 2.1 kb in size, which carries the aecD gene and adjacent regions.

The amplified DNA fragment of approx. 2.1 kb in length is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

The DNA fragment purified is cleaved with the restriction enzyme BamHI and EcoRV (Amersham Pharmacia, Freiburg, Germany). The ligation of the fragment in the vector pUC18 then takes place (Norrander et al., Gene 26:101-106 (1983)). This is cleaved beforehand with the restriction enzymes BglII and SmaI, dephosphorylated, mixed with the aecD-carrying fragment of approx. 1.5 kb, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany). The ligation batch is transformed in the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands). Selection of plasmid-carrying cells is made by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal (64 mg/l).

The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The resulting plasmid is called pUC18aecD.

Plasmid DNA was isolated from the strain DSM14242 (see Example 1.1) which carries the plasmid pCRIITOPOlysC and cleaved with the restriction enzyme BamHI (Amersham-Pharmacia, Freiburg, Germany) and then treated with Klenow polymerase. After separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the $lysC^{FBR}$-containing DNA fragment approx. 1.7 kb in length is isolated from the agarose gel and employed for ligation with the vector pUC18aecD described above. This is cleaved beforehand with the restriction enzyme StuI, dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim, Germany), mixed with the $lysC^{FBR}$ fragment of approx. 1.7 kb and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5αmcr (Life Technologies GmbH, Karlsruhe, Germany) is then transformed with the ligation batch (Hanahan, In: DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pUC18aecD1.

The plasmid pUC18aecD1 is cleaved with the restriction enzyme KpnI and then treated with Klenow polymerase. The plasmid is then cleaved with the restriction enzyme SalI (Amersham-Pharmacia, Freiburg, Germany) and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the fragment of approx. 3.2 kb which carries aecD and lysC is isolated from the agarose gel and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al. (Gene 14: 69-73 (1994)). This is cleaved beforehand with the restriction enzymes SmaI and SalI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the fragment of approx. 3.2 kb which carries aecD and lysC, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation

batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBaecD1_1. A map of the plasmid is shown in Figure 2.

The plasmid pK18mobsacBaecD1_1 was deposited in the form of a pure culture of the strain E. coli DH5αmcr/pK18mobsacBaecD1_1 (= DH5alphamcr/pK18mobsacBaecD1_1) on 5th June 2002 under number DSM15040 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) in accordance with the Budapest Treaty.

1.8 Incorporation of a second copy of the lysC gene as the $lysC^{FBR}$ allele into the chromosome (target site: aecD gene) of the strain DSM12866 by means of the replacement vector pK18mobsacBaecD1_1

As described in Example 1.3, the plasmid pK18mobsacBaecD1_1 described in Example 1.4 is transferred into the C. glutamicum strain DSM12866 by conjugation. Selection is made for targeted recombination events in the chromosome of C. glutamicum DSM12866 as described in Example 1.3.

Depending on the position of the second recombination event, after the excision the second copy of the $lysC^{FBR}$ allele manifests itself in the chromosome at the aecD locus, or the original aecD locus of the host remains.

Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". Approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin"

are investigated with the aid of the polymerase chain reaction. A DNA fragment which carries the aecD gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The same primer oligonucleotides as are described in Example 1.7 for the construction of the integration plasmid are chosen for the PCR.

aecD_beg (SEQ ID NO: 11):
5` GAA CTT ACG CCA AGC TGT TC 3`

aecD_end (SEQ ID NO: 12):
5` AGC ACC ACA ATC AAC GTG AG 3`

The primers allow amplification of a DNA fragment approx. 2.1 kb in size in control clones with the original aecD locus. In clones with a second copy of the $lysC^{FBR}$ allele in the chromosome at the aecD locus, DNA fragments with a size of approx. 3.8 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel.

A clone which, in addition to the copy of the wild-type gene present at the lysC locus, has a second copy of the lysC gene in the form of the $lysC^{FBR}$ allele lysC T311I at the aecD locus in the chromosome was identified in this manner. This clone was called strain DSM12866aecD::lysC.

Example 2

Incorporation of a second copy of the ddh gene into the chromosome (target site: gluB gene) of the strain DSM12866

2.1 Construction of the replacement vector pK18mobsacBglu2_1

The Corynebacterium glutamicum strain ATCC13032 is used as the donor for the chromosomal DNA. From the strain ATCC13032, chromosomal DNA is isolated using the conventional methods (Eikmanns et al., Microbiology 140:

1817 - 1828 (1994)). With the aid of the polymerase chain reaction, a DNA fragment which carries the gluB gene and surrounding regions is amplified. On the basis of the sequence of the gluABCD gene cluster known for C. glutamicum (Kronemeyer et al., Journal of Bacteriology, 177: 1152 - 1158 (1995); EP1108790) (Accession Number X81191 and AX127149), the following primer oligonucleotides are chosen for the PCR:

gluA_beg (SEQ ID NO: 13):
5` CAC GGT TGC TCA TTG TAT CC 3`

gluD_end (SEQ ID NO: 14):
5` CGA GGC GAA TCA GAC TTC TT 3`

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx 4.4 kb in size, which carries the gluB gene and surrounding regions.

The amplified DNA fragment is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

Ligation of the fragment is then carried out by means of the TOPO TA Cloning Kit (Invitrogen, Leek, The Netherlands, Cat. Number K4600-01) in the vector pCRII-TOPO. The ligation batch is transformed in the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands). Selection of plasmid-carrying cells is made by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal (64 mg/l).

The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in

agarose gel. The resulting plasmid is called pCRII-TOPOglu2.

The plasmid pCRII-TOPOglu2 is cleaved with the restriction enzymes EcoRI and SalI (Amersham-Pharmacia, Freiburg, Germany) and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the gluB fragment of approx. 3.7 kb is isolated from the agarose gel and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al. (Gene 14, 69-73 (1994)). This is cleaved beforehand with the restriction enzymes EcoRI and SalI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the gluB fragment of approx. 3.7 kb, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli Strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBglu2.

As described in Example 2.1, a DNA fragment which carries the ddh gene and surrounding regions is also amplified with the aid of the polymerase chain reaction. On the basis of the sequence of the ddh gene cluster known for C. glutamicum (Ishino et al., Nucleic Acids Research 15,

3917(1987)) (Accession Number Y00151), the following primer oligonucleotides are chosen for the PCR:

ddh_beg (SEQ ID NO: 15):
5` CTG AAT CAA AGG CGG ACA TG 3`

ddh_end (SEQ ID NO: 16):
5` TCG AGC TAA ATT AGA CGT CG 3`

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx 1.6 kb in size, which carries the ddh gene.

The amplified DNA fragment of approx. 1.6 kb in length, which the ddh gene, is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

After purification, the fragment carrying the ddh gene is employed for ligation in the vector pK18mobsacBglu2 described. This is partly cleaved beforehand with the restriction enzyme BamHI. By treatment of the vector with a Klenow polymerase (Amersham-Pharmacia, Freiburg, Germany), the overhangs of the cleaved ends are completed to blunt ends, the vector is then mixed with the DNA fragment of approx. 1.6 kb which carries the ddh gene and the mixture is treated with T4 DNA ligase (Amersham-Pharmacia, Freiburg, Germany). By using Vent Polymerase (New England Biolabs, Frankfurt, Germany) for the PCR reaction, a ddh-carrying DNA fragment which has blunt ends and is suitable for ligation in the pretreated vector pK18mobsacBglu2 is generated.

The E. coli strain DH5αmcr (Life Technologies GmbH, Karlsruhe, Germany) is then transformed with the ligation

batch (Hanahan, In: DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBglu2_1. A map of the plasmid is shown in Figure 4.

2.2 Incorporation of a second copy of the ddh gene into the chromosome (target site: gluB gene) of the strain DSM12866 by means of the replacement vector pK18mobsacBglu2_1

As described in Example 1.3, the plasmid pK18mobsacBglu2_1 described in Example 2.1 is transferred into the C. glutamicum strain DSM12866 by conjugation. Selection is made for targeted recombination events in the chromosome of C. glutamicum DSM12866 as described in Example 1.3. Depending on the position of the second recombination event, after the excision the second copy of the ddh gene manifests itself in the chromosome at the gluB locus, or the original gluB locus of the host remains.

Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". Approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin" are investigated with the aid of the polymerase chain reaction. A DNA fragment which carries the glu region described is amplified here from the chromosomal DNA of the colonies. The same primer oligonucleotides as are described

in Example 2.1 for the construction of the replacement plasmid are chosen for the PCR.

gluA_beg (SEQ ID NO: 13):
5` CAC GGT TGC TCA TTG TAT CC 3`

gluD_end (SEQ ID NO: 14):
5` CGA GGC GAA TCA GAC TTC TT 3`

The primers allow amplification of a DNA fragment approx. 4.4 kb in size in control clones with the original glu locus. In clones with a second copy of the ddh gene in the chromosome at the gluB locus, DNA fragments with a size of approx. 6 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel.

A clone which, in addition to the copy present at the ddh locus, has a second copy of the ddh gene at the gluB locus in the chromosome was identified in this manner. This clone was called strain DSM12866glu::ddh.

Example 3

Incorporation of a second copy of the dapA gene into the chromosome (target site: aecD gene) of the strain DSM12866

3.1 Construction of the replacement vector pK18mobsacBaecD2_1

The Corynebacterium glutamicum strain ATCC13032 is used as the donor for the chromosomal DNA. From the strain ATCC13032, chromosomal DNA is isolated using the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). With the aid of the polymerase chain reaction, a DNA fragment which carries the aecD gene and surrounding regions is amplified. On the basis of the sequence of the aecD gene known for C. glutamicum (Rossol et al., Journal of Bacteriology 174:2968-2977 (1992))

(Accession Number M89931), the following primer oligonucleotides are chosen for the PCR:

aecD_beg (SEQ ID NO: 11):
5` GAA CTT ACG CCA AGC TGT TC 3`

aecD_end (SEQ ID NO: 12):
5` AGC ACC ACA ATC AAC GTG AG 3`

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx 2.1 kb in size, which carries the aecD gene and adjacent regions.

The amplified DNA fragment of approx. 2.1 kb in length is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

The DNA fragment purified is cleaved with the restriction enzyme BglII and EcoRV (Amersham Pharmacia, Freiburg, Germany). The ligation of the fragment in the vector pUC18 then takes place (Norrander et al., Gene 26:101-106 (1983)). This is cleaved beforehand with the restriction enzymes BamHI and SmaI and dephosphorylated, mixed with the aecD-carrying fragment of approx. 1.5 kb, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany). The ligation batch is transformed in the E. coli strain TOP10 (Invitrogen, Leek, The Netherlands). Selection of plasmid-carrying cells is made by plating out the transformation batch on kanamycin (50 mg/l)-containing LB agar with X-Gal (64 mg/l).

The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The resulting plasmid is called pUC18aecD.

With the aid of the polymerase chain reaction, a further DNA fragment which carries the dapA gene and surrounding regions is amplified. On the basis of the sequence of the dapA gene known for C. glutamicum (Bonassi et al., Nucleic Acids Research 18:6421 (1990)) (Accession Number X53993 and AX127149), the following primer oligonucleotides are chosen for the PCR:

dapA_beg (SEQ ID NO: 17):
5` CGA GCC AGT GAA CAT GCA GA 3`

dapA_end (SEQ ID NO: 18):
5` CTT GAG CAC CTT GCG CAG CA 3`

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx. 1.4 kb in size, which carries the dapA gene and adjacent regions.

The amplified DNA fragment of approx. 1.4 kb in length is identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

After purification, the dapA-containing DNA fragment approx. 1.4 kb in length is employed for ligation with the vector pUC18aecD described above. This is cleaved beforehand with the restriction enzyme StuI, mixed with the DNA fragment of approx. 1.4 kb, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5αmcr (Life Technologies GmbH, Karlsruhe, Germany) is then transformed with the ligation batch (Hanahan, In: DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al.,

Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pUC18aecD2.

The plasmid pUC18aecD2 is cleaved with the restriction enzyme SalI and partly with EcoRI (Amersham-Pharmacia, Freiburg, Germany) and after separation in an agarose gel (0.8%) with the aid of the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) the fragment of approx. 2.7 kb which carries aecD and dapA is isolated from the agarose gel and employed for ligation with the mobilizable cloning vector pK18mobsacB described by Schäfer et al. (Gene 14: 69-73 (1994)). This is cleaved beforehand with the restriction enzymes EcoRI and with SalI and dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim), mixed with the fragment of approx. 2.7 kb which carries aecD and dapA, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5α (Grant et al.; Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) is then transformed with the ligation batch (Hanahan, In. DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which is supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel

electrophoresis. The plasmid is called pK18mobsacBaecD2_1. A map of the plasmid is shown in Figure 5.

3.2 Incorporation of a second copy of the dapA gene into the chromosome (target site: aecD gene) of the strain DSM12866 by means of the replacement vector pK18mobsacBaecD2_1

As described in Example 1.3, the plasmid pK18mobsacBaecD2_1 described in Example 3.1 is transferred into the C. glutamicum strain DSM12866 by conjugation. Selection is made for targeted recombination events in the chromosome of C. glutamicum DSM12866 as described in Example 1.3. Depending on the position of the second recombination event, after the excision the second copy of the dapA gene manifests itself in the chromosome at the aecD locus, or the original aecD locus of the host remains.

Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". Approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin" are investigated with the aid of the polymerase chain reaction. A DNA fragment which carries the aecD gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The same primer oligonucleotides as are described in Example 3.1 for the construction of the integration plasmid are chosen for the PCR.

aecD_beg (SEQ ID NO: 11):
5` GAA CTT ACG CCA AGC TGT TC 3`

aecD_end (SEQ ID NO: 12):
5` AGC ACC ACA ATC AAC GTG AG 3`

The primers allow amplification of a DNA fragment approx. 2.1 kb in size in control clones with the original aecD locus. In clones with a second copy of the dapA gene in the

chromosome at the aecD locus, DNA fragments with a size of approx. 3.6 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel.

A clone which, in addition to the copy present at the dapA locus, has a second copy of the dapA gene at the aecD locus in the chromosome was identified in this manner. This clone was called strain DSM12866aecD::dapA.

Example 4

Incorporation of a second copy of the pyc gene in the form of the pyc allele pycP458S into the chromosome (target site: pck gene) of the strain DSM12866

4.1 Construction of the replacement vector pK18mobsacBpck1_3

The replacement vector pK18mobsacBpck1 described in Example 1.5 is used as the base vector for insertion of the pyc allele.

As described in Example 2.1, a DNA fragment which carries the pyc gene and surrounding regions is also amplified with the aid of the polymerase chain reaction. On the basis of the sequence of the pyc gene cluster known for C. glutamicum (Peters-Wendisch et al., Journal of Microbiology 144: 915-927 (1998)) (Accession Number Y09548), the following primer oligonucleotides are chosen for the PCR:

pyc_beg (SEQ ID NO: 19):
5` TC(A CGC GT)C TTG AAG TCG TGC AGG TCA G 3`

pyc_end (SEQ ID NO: 20):
5` TC(A CGC GT)C GCC TCC TCC ATG AGG AAG A 3`

The primers shown are synthesized by MWG Biotech and the PCR reaction is carried out by the standard PCR method of

Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA fragment of approx 3.6 kb in size, which carries the pyc gene. The primers moreover contain the sequence for the cleavage site of the restriction endonuclease MluI, which is marked by parentheses in the nucleotide sequence shown above.

The amplified DNA fragment of approx. 3.6 kb in length, which carries the pyc gene, is cleaved with the restriction endonuclease MluI, identified by means of electrophoresis in a 0.8% agarose gel and isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

After purification, the fragment carrying the pyc gene is employed for ligation in the vector pK18mobsacBpck1 described. This is cleaved beforehand with the restriction enzyme BssHII, dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim, Germany), mixed with the DNA fragment of approx. 3.6 kb which carries the pyc gene, and the mixture is treated with T4 DNA Ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain DH5αmcr (Life Technologies GmbH, Karlsruhe, Germany) is then transformed with the ligation batch (Hanahan, In: DNA Cloning. A Practical Approach. Vol. 1, ILR-Press, Cold Spring Harbor, New York, 1989).

Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Ed., Cold Spring Harbor, New York, 1989), which was supplemented with 50 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction cleavage and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacBpck1_2.

4.2 Construction of the pyc allele pyc P458S by means of site-specific mutagenesis of the wild-type pyc gene

The site-directed mutagenesis is carried out with the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA). EP-A-1108790 describes a point mutation in the pyc gene for C. glutamicum which allows improved L-lysine production. On the basis of the point mutation in the nucleotide sequence of cytosine to thymine in the pyc gene at position 1372, replacement in the amino acid sequence derived therefrom of proline for serine at position 458 results. The allele is called pyc P458S. To generate the mutation described, the following primer oligonucleotides are chosen for the linear amplification:

P458S-1 (SEQ ID NO: 21):
5′ GGATTCATTGCCGATCAC (TCG) CACCTCCTTCAGGCTCCA 3′

P458S-2 (SEQ ID NO: 22):
5′GTGGAGGAAGTCCGAGGT (CGA) GTGATCGGCAATGAATCC 3′

The primers shown are synthesized by MWG Biotech. The codon for serine, which is to replace the proline at position 458, is marked by parentheses in the nucleotide sequence shown above. The plasmid pK18mobsacBpck1_2 described in Example 4.1 is employed with the two primers, which are each complementary to a strand of the plasmid, for linear amplification by means of Pfu Turbo DNA polymerase. By this lengthening of the primers, a mutated plasmid with broken circular strands is formed. The product of the linear amplification is treated with DpnI - this endonuclease cleaves the methylated and half-methylated template DNA specifically. The newly synthesized broken, mutated vector DNA is transformed in the E. coli strain XL1 Blue (Bullock, Fernandez and Short, BioTechniques (5) 376-379 (1987)). After the transformation, the XL1 Blue cells repair the breaks in the mutated plasmids. Selection of the transformants was carried out on LB medium with kanamycin

50 mg/l. The plasmid obtained is checked by means of restriction cleavage, after isolation of the DNA, and identified in agarose gel. The DNA sequence of the mutated DNA fragment is checked by sequencing. The sequence of the PCR product coincides with the sequence described Ohnishi et al. (2002). The resulting plasmid is called pK18mobsacBpck1_3. A map of the plasmid is shown in Figure 6.

4.3 Incorporation of a second copy of the pyc gene in the form of the pyc allele pycP458S into the chromosome (target site pck gene) of the strain DSM12866 by means of the replacement vector pk18mobsacBpck1_3

The plasmid pK18mobsacBpck1_3 described in Example 4.2 is transferred as described in Example 1.3 into the C. glutamicum strain DSM12866 by conjugation. Selection is made for targeted recombination events in the chromosome of C. glutamicum DSM12866 as described in Example 1.3. Depending on the position of the second recombination event, after the excision the second copy of the pyc allele manifests itself in the chromosome at the pck locus, or the original pck locus of the host remains.

Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". Approximately 20 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin" are investigated with the aid of the polymerase chain reaction. A DNA fragment which carries the pck gene and surrounding regions is amplified here from the chromosomal DNA of the colonies. The same primer oligonucleotides as are described in Example 1.5 for the construction of the replacement plasmid are chosen for the PCR.

pck_beg (SEQ ID NO: 9):
5` TA(A GAT CT) G CCG GCA TGA CTT CAG TTT 3`

pck_end (SEQ ID NO: 10):
5` AC(A GAT CT) G GTG GGA GCC TTT CTT GTT ATT3`

The primers allow amplification of a DNA fragment approx. 2.9 kb in size in control clones with the original pck locus. In clones with a second copy of the pyc allele in the chromosome at the pck locus, DNA fragments with a size of approx. 6.5 kb are amplified.

The amplified DNA fragments are identified by means of electrophoresis in a 0.8% agarose gel.

A clone which, in addition to the copy of the wild-type gene present at the pyc locus, has a second copy of the pyc gene in the form of the pyc allele pycP458S at the pck locus in the chromosome was identified in this manner. This clone was called strain DSM12866pck::pyc.

Example 5

Preparation of Lysine

The C. glutamicum strains DSM13994glu::lysC, DSM12866glu::lysC, DSM12866pck::lysC, DSM12866aecD::lysC, DSM12866glu::ddh, DSM12866aecD::dapA and DSM12866pck::pyc obtained in Example 1, 2, 3 and 4 are cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined.

For this, the cultures are first incubated on a brain-heart agar plate (Merck, Darmstadt, Germany) for 24 hours at 33ºC. Starting from this agar plate culture, a preculture is seeded (10 ml medium in a 100 ml conical flask). The medium MM is used as the medium for the preculture. The preculture is incubated for 24 hours at 33ºC at 240 rpm on a shaking machine. A main culture is seeded from this preculture such that the initial OD (660 nm) of the main

culture is 0.1 OD. The Medium MM is also used for the main culture.

Medium MM

CSL	5 g/l
MOPS	20 g/l
Glucose (autoclaved separately)	50 g/l
Salts:	
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l
$CaCl_2 * 2 H_2O$	10 mg/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0 mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
$CaCO_3$	25 g/l

The CSL (corn steep liquor), MOPS (morpholinopropanesulfonic acid) and the salt solution are brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions, as well as the $CaCO_3$ autoclaved in the dry state, are then added.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Culturing is carried out at 33ºC and 80% atmospheric humidity.

After 48 hours, the OD is determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed is determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivation with ninhydrin detection.

The result of the experiment is shown in Table 10.

Table 10

Strain	OD (660 nm)	Lysine HCl g/l
DSM13994	12.0	19.1
DSM13994glu::lysC	9.9	20.0
DSM12866	12.5	14.9
DSM15039	11.4	16.2
DSM12866pck::lysC	12.6	16.5
DSM12866aecD::lysC	12.0	15.9
DSM12866glu::ddh	11.0	15.5
DSM12866aecD::dapA	11.1	16.2
DSM12866pck::pyc	10.9	16.9

Brief Description of the Figures:

The base pair numbers stated are approximate values obtained in the context of reproducibility of measurements.

Figure 1: Map of the plasmid pK18mobsacBglu1_1.

The abbreviations and designations used have the following meaning:

KanR: Kanamycin resistance gene

HindIII: Cleavage site of the restriction enzyme HindIII

BamHI: Cleavage site of the restriction enzyme BamHI

lysC: $lysC^{FBR}$ allele, lysC T311I

'gluA: 3' terminal fragment of the gluA gene

gluB': 5' terminal fragment of the gluB gene

'gluB: 3' terminal fragment of the gluB gene

gluC': 5' terminal fragment of the gluC gene

sacB: sacB gene

RP4mob: mob region with the replication origin for the transfer (oriT)

oriV: Replication origin V

Figure 2: Map of the plasmid pK18mobsacBaecD1_1.

The abbreviations and designations used have the following meaning:

KanR: Kanamycin resistance gene

SalI: Cleavage site of the restriction enzyme SalI

lysC: $lysC^{FBR}$ allele, lysC T311I

aecD': 5' terminal fragment of the aecD gene

'aecD: 3' terminal fragment of the aecD gene

sacB: sacB gene

RP4mob: mob region with the replication origin for the transfer (oriT)

oriV: Replication origin V

Figure 3: Map of the plasmid pK18mobsacBpck1_1.

The abbreviations and designations used have the following meaning:

KanR:	Kanamycin resistance gene
BamHI:	Cleavage site of the restriction enzyme BamHI
lysC:	$lysC^{FBR}$ allele, lysC T311I
pck':	5' terminal fragment of the pck gene
'pck:	3' terminal fragment of the pck gene
sacB:	sacB gene
RP4mob:	mob region with the replication origin for the transfer (oriT)
oriV:	Replication origin V

Figure 4: Map of the plasmid pK18mobsacBgluB2_1.

The abbreviations and designations used have the following meaning:

KanR:	Kanamycin resistance gene
SalI	Cleavage site of the restriction enzyme SalI
EcoRI	Cleavage site of the restriction enzyme EcoRI
BamHI:	Cleavage site of the restriction enzyme BamHI
ddh:	ddh gene

gluA	gluA gene
gluB':	5' terminal fragment of the gluB gene
'gluB:	3' terminal fragment of the gluB gene
gluC	gluC gene
gluD':	5' terminal fragment of the gluD gene
sacB:	sacB gene
RP4mob:	mob region with the replication origin for the transfer (oriT)
oriV:	Replication origin V

Figure 5: Map of the plasmid pK18mobsacBaecD2_1.

The abbreviations and designations used have the following meaning:

KanR:	Kanamycin resistance gene
EcoRI	Cleavage site of the restriction enzyme EcoRI
SalI:	Cleavage site of the restriction enzyme SalI
dapA:	dapA gene
aecD':	5' terminal fragment of the aecD gene
'aecD:	3' terminal fragment of the aecD gene
sacB:	sacB gene
RP4mob:	mob region with the replication origin for the transfer (oriT)
oriV:	Replication origin V

Figure 6: Map of the plasmid pK18mobsacBpck1_3.

The abbreviations and designations used have the following meaning:

KanR:	Kanamycin resistance gene
pyc:	pyc allele, pyc P458S
pck':	5' terminal fragment of the pck gene
'pck:	3' terminal fragment of the pck gene
sacB:	sacB gene
RP4mob:	mob region with the replication origin for the transfer (oriT)
oriV:	Replication origin V

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

DSMZ
Deutsche Sammlung von
Mikroorganismen und
Zellkulturen GmbH

INTERNATIONAL FORM

Degussa AG

Kantstr. 2

33790 Halle (Westf.)

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
DSM12866glu::lysC	DSM 15039

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

(x) a scientific description
(x) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-06-05 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit)
and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Weis Date: 2002-06-06

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 12/2001

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG

Kantstr. 2

33790 Halle (Westf.)

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa AG Kantstr. 2 Address: 33790 Halle (Westf.)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15039 Date of the deposit or the transfer[1]: 2002-06-05

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2002-06-05
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): V. Weiß Date: 2002-06-06

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
[2] In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
[3] Mark with a cross the applicable box.
[4] Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 12/2001

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2
33790 Halle (Westf.)

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
DH5alphamcr/pK18mobsacBaecD1_1	DSM 15040

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

(X) a scientific description
(X) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2002-06-05 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit)
and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): i. Weiss Date: 2002-06-06

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

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DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2
33790 Halle (Westf.)

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa AG Kantstr. 2 Address: 33790 Halle (Westf.)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15040 Date of the deposit or the transfer[1]: 2002-06-05

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2002-06-05
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): V. Weiss Date: 2002-06-06

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
[2] In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
[3] Mark with a cross the applicable box.
[4] Fill in if the information has been requested and if the results of the test were negative.

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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2
33790 Halle/Künsebeck

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
Issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
DH5alphamcr/ pK18mobsacBglu1_1	DSM 14243

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

(X) a scientific description
(X) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2001-04-20 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Weiss Date: 2001-04-26

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

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BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2
33790 Halle/Künsebeck

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Degussa AG Kantstr. 2 Address: 33790 Halle/Künsebeck	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 14243 Date of the deposit or the transfer[1]: 2001-04-20

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2001-04-20 [2].
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): V. Weiks Date: 2001-04-26

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
[2] In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
[3] Mark with a cross the applicable box.
[4] Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 0196

What is claimed is:

1. Coryneform bacteria which produce chemical compounds, wherein these have, in addition to at least one copy, present at the natural site (locus), of an open reading frame (ORF), gene or allele which codes for the synthesis of a protein or an RNA, a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question at a second, optionally third or fourth site in a form integrated into the chromosome, no nucleotide sequence which is capable of/enables episomal replication or transposition in microorganisms and no nucleotide sequence(s) which impart(s) resistance to antibiotics being present at the second, optionally third or fourth site, and the second, optionally third or fourth site not relating to open reading frames (ORF), genes or alleles which are essential for the growth of the bacteria and the production of the desired compound.

2. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the coryneform bacteria belong to the genus Corynebacterium.

3. Coryneform bacteria of the genus Corynebacterium according to claim 2 which produce chemical compounds, wherein these belong to the species Corynebacterium glutamicum.

4. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the chemical compound is a compound chosen from the group consisting of L-amino acids, vitamins, nucleosides and nucleotides.

5. Coryneform bacteria according to claim 1 which produce chemical compounds, wherein the chemical compound is one or more L-amino acids chosen from the group consisting of L-aspartic acid, L-asparagine, L-

threonine, L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and L-arginine.

6. Coryneform bacteria according to claims 1 and 4 which produce chemical compounds, wherein the L-amino acid is L-lysine, and these bacteria have, in addition to at least one copy of an open reading frame (ORF), gene or allele of lysine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele of lysine production in question at in each case a second, optionally third or fourth site in a form integrated into the chromosome.

7. Coryneform bacteria according to claim 6 which produce L-lysine, wherein the coryneform bacteria belong to the genus Corynebacterium.

8. Coryneform bacteria of the genus Corynebacterium according to claim 7 which produce L-lysine, wherein these belong to the species Corynebacterium glutamicum.

9. Coryneform bacteria according to claim 6 which produce L-lysine, wherein the open reading frame (ORF), gene or allele of lysine production is one or more open reading frame(s), one or more gene(s) or allele(s) chosen from the group consisting of accBC, accDA, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dapA, dapB, dapC, dapD, dapE, dapF, ddh, dps, eno, gap, gap2, gdh, gnd, lysC, $lysC^{FBR}$, lysE, msiK, opcA, oxyR, ppc, ppc^{FBR}, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T.

10. Coryneform bacteria according to claim 6 which produce L-lysine, wherein the open reading frame, gene or allele of lysine production is one or more gene(s) or allele(s) chosen from the group consisting of dapA, ddh, $lysC^{FBR}$ and pyc P458S.

11. Coryneform bacteria according to claim 6 which produce L-lysine, wherein the open reading frame, gene or allele of lysine production is a $lysC^{FBR}$ allele which codes for a feed back resistant form of aspartate kinase.

12. Coryneform bacteria according to claim 11 which produce L-lysine, wherein the feed back resistant form of aspartate kinase coded by the $lysC^{FBR}$ allele contains an amino acid sequence according to SEQ ID NO:2, SEQ ID NO:2 containing one or more amino acid replacements chosen from the group consisting of A279T, A279V, S301F, T308I, S301Y, G345D, R320G, T311I and S381F.

13. Coryneform bacteria according to claim 11 which produce L-lysine, wherein the feed back resistant form of aspartate kinase coded by the $lysC^{FBR}$ allele includes an amino acid sequence according to SEQ ID NO:4.

14. Coryneform bacteria according to claim 11 which produce L-lysine, wherein the coding region of the $lysC^{FBR}$ allele includes the nucleotide sequence of SEQ ID NO:3.

15. Coryneform bacteria according to claim 6 which produce L-lysine, wherein the particular second, optionally third or fourth site is a gene chosen from the group consisting of aecD, ccpA1, ccpA2, citA, citB, citE, fda, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, mqo, pck, pgi and poxB.

16. Coryneform bacteria according to claim 6 which produce L-lysine, wherein the particular second, optionally third or fourth site is a site chosen from the group

consisting of intergenic regions of the chromosome, prophages contained in the chromosome and defective phages contained in the chromosome.

17. Coryneform bacteria according to claim 15 which produce L-lysine, wherein the particular second, optionally third or fourth site is the aecD gene site.

18. Coryneform bacteria according to claim 15 which produce L-lysine, wherein the particular second, optionally third or fourth site is the gluB gene site.

19. Coryneform bacteria according to claim 15 which produce L-lysine, wherein the particular second, optionally third or fourth site is the pck gene site.

20. Process for the preparation of chemical compounds by fermentation of coryneform bacteria, in which the following steps are carried out:

a) fermentation of coryneform bacteria, which

a1) which have, in addition to at least one copy, present at the natural site (locus), of an open reading frame (ORF), gene or allele which codes for the synthesis of a protein or an RNA, a second, optionally third or fourth copy of this open reading frame (ORF), gene or allele at a second, optionally third or fourth site in a form integrated into the chromosome, no nucleotide sequence which is capable of/enables episomal replication or transposition in microorganisms and no nucleotide sequence(s) which impart(s) resistance to antibiotics being present at the second, optionally third or fourth site, and the second, optionally third or fourth site not relating to open reading frames (ORF), genes or alleles which are essential for the growth of the

bacteria and the production of the desired compound, and

a2) in which the intracellular activity of the corresponding protein is increased, in particular the nucleotide sequence which codes for this protein is over-expressed,

c) concentration of the chemical compound(s) in the fermentation broth and/or in the cells of the bacteria,

d) isolation of the chemical compound(s), optionally

e) with constituents from the fermentation broth and/or the biomass to the extent of > (greater than) 0 to 100 wt.%.

21. Process according to claim 20, wherein the coryneform bacteria belong to the genus Corynebacterium.

22. Process according to claim 20, wherein the coryneform bacteria of the genus Corynebacterium belong to the species Corynebacterium glutamicum.

23. Process according to claim 20, wherein the chemical compound is a compound chosen from the group consisting of L-amino acids, vitamins, nucleosides and nucleotides.

24. Process according to claim 20, wherein the chemical compound is one or more L-amino acids chosen from the group consisting of L-aspartic acid, L-asparagine, L-threonine, L-serine, L-glutamic acid, L-glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and L-arginine.

25. Process according to claim 24, wherein the chemical compound is L-lysine.

26. Process for the preparation of L-lysine, which comprises the following steps:

a) fermentation of coryneform bacteria which have, in addition to at least one copy of an open reading frame (ORF), gene or allele of lysine production present at the natural site (locus), in each case a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele of lysine production in question at in each case a second, optionally third or fourth site in a form integrated into the chromosome

under conditions which allow expression of the said open reading frames (ORF), genes or alleles mentioned.

27. Process for the preparation of L-lysine according to claim 26, wherein the open reading frame (ORF), gene or allele of lysine production is an open reading frame, a gene or allele chosen from the group consisting of accBC, accDA, cstA, cysD, cysE, cysH, cysK, cysN, cysQ, dapA, dapB, dapC, dapD, dapE, dapF, ddh, dps, eno, gap, gap2, gdh, gnd, lysC, $lysC^{FBR}$, lysE, msiK, opcA, oxyR, ppc, ppc^{FBR}, pgk, pknA, pknB, pknD, pknG, ppsA, ptsH, ptsI, ptsM, pyc, pyc P458S, sigC, sigD, sigE, sigH, sigM, tal, thyA, tkt, tpi, zwa1, zwf and zwf A213T.

28. Process for the preparation of L-lysine according to claim 26, wherein the open reading frame (ORF), gene or allele of lysine production is a gene or allele chosen from the group consisting of dapA, ddh, $lysC^{FBR}$ and pyc P458S.

29. Process for the preparation of L-lysine according to claim 26, wherein the open reading frame (ORF), gene or allele of lysine production is a $lysC^{FBR}$ allele which codes for a feed back resistant form of aspartate kinase.

30. Process for the preparation of L-lysine according to claim 29, wherein the feed back resistant form of aspartate kinase coded by the $lysC^{FBR}$ allele contains an amino acid sequence according to SEQ ID NO:2, SEQ ID NO:2 containing one or more amino acid replacements chosen from the group consisting of A279T, A279V, S301F, T308I, S301Y, G345D, R320G, T311I and S381F.

31. Process for the preparation of L-lysine according to claim 29, wherein the feed back resistant form of aspartate kinase coded by the $lysC^{FBR}$ allele includes an amino acid sequence according to SEQ ID NO:4.

32. Process for the preparation of L-lysine according to claim 29, wherein the coding region of the $lysC^{FBR}$ allele includes the nucleotide sequence of SEQ ID NO:3.

33. Process for the preparation of L-lysine according to claim 26, wherein the particular second, optionally third or fourth site is a site chosen from the group consisting of aecD, ccpA1, ccpA2, citA, citB, citE, fda, gluA, gluB, gluC, gluD, luxR, luxS, lysR1, lysR2, lysR3, menE, mqo, pck, pgi and poxB.

34. Process for the preparation of L-lysine according to claim 26, wherein the second, optionally third or fourth site is the aecD gene site.

35. Process for the preparation of L-lysine according to claim 26, wherein the second, optionally third or fourth site is the gluB gene site.

36. Process for the preparation of L-lysine according to claim 26, wherein the second, optionally third or fourth site is the pck gene site.

37. Process for the production of coryneform bacteria which produce one or more chemical compounds, which comprises

a) isolating the nucleotide sequence of at least one desired ORF, gene or allele which codes for a protein or an RNA, optionally including the expression and/or regulation signals, preferably from coryneform bacteria,

b) providing the 5' and the 3' end of the ORF, gene or allele with nucleotide sequences of the target site,

c) preferably incorporating the nucleotide sequence of the desired ORF, gene or allele provided with nucleotide sequences of the target site into a vector which does not replicate or replicates to only a limited extent in coryneform bacteria,

d) transferring the nucleotide sequences according to b) or c) into coryneform bacteria, and

e) isolating coryneform bacteria in which the nucleotide sequence(s) according to a) is incorporated at the target site, no nucleotide sequence(s) which is(are) capable of/enable(s) episomal replication or transposition in microorganisms, and no nucleotide sequence(s) which impart(s) resistance to antibiotics remaining at the target site.

38. Plasmid pK18mobsacBglu1_1 shown in Figure 1 and deposited in the form of a pure culture of the strain E. coli DH5αmcr/pK18mobsacBglu1_1 (= DH5alpha mcr/pK18mobsacBglu1_1) under number DSM14243.

39. Plasmid pK18mobsacBaecD1_1 shown in Figure 2 and deposited in the form of a pure culture of the strain E. coli DH5αmcr/pK18mobsacBaecD1_1 (= DH5alphamcr/pK18mobsacBaecD1_1) under number DSM15040.

40. Corynebacterium glutamicum strain DSM12866glu::lysC deposited in the form of a pure culture under number DSM15039.

Figure 1: Plasmid pK18mobsacBglu1_1

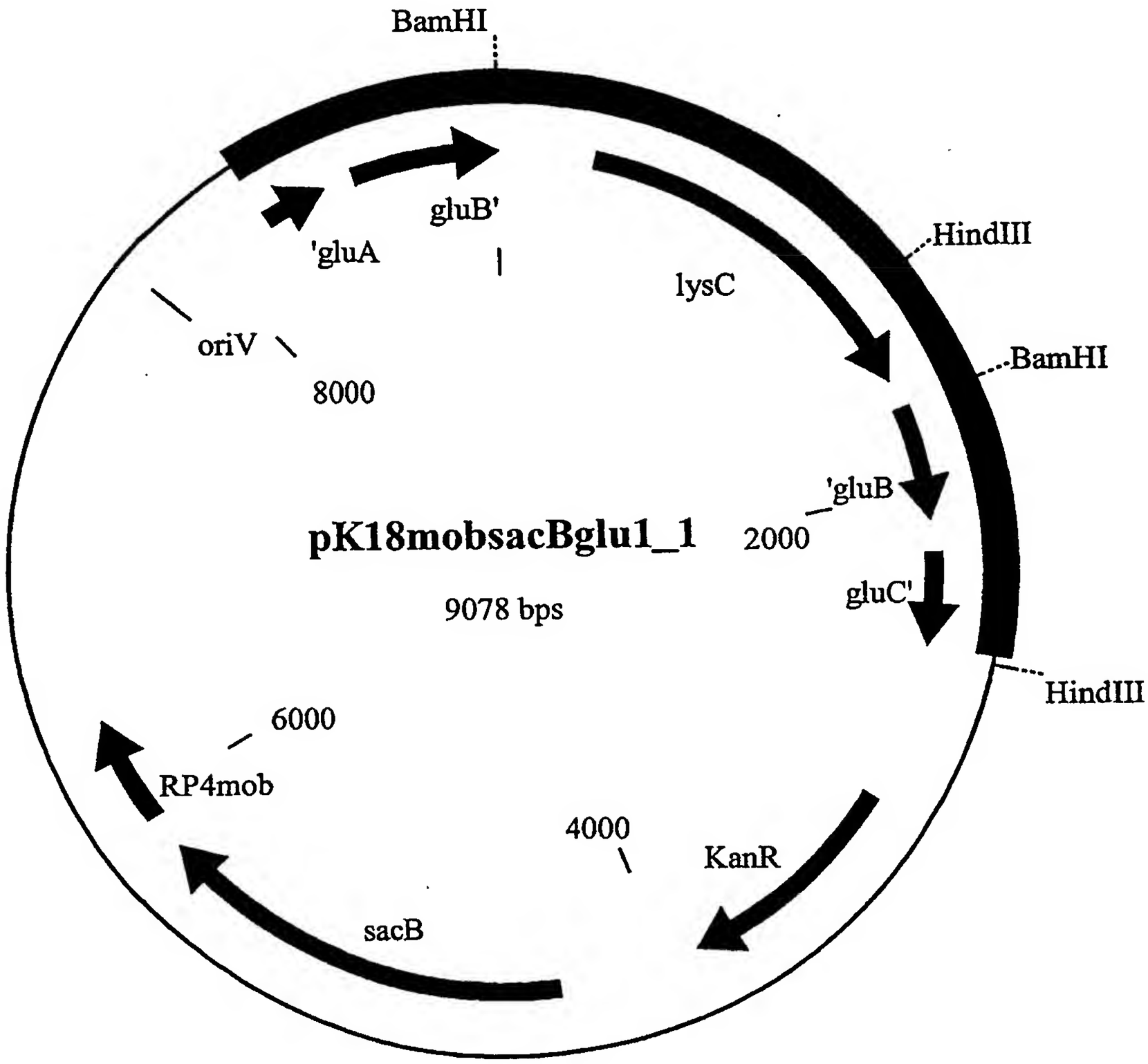


Figure 2: Plasmid pK18mobsacBaecD1_1

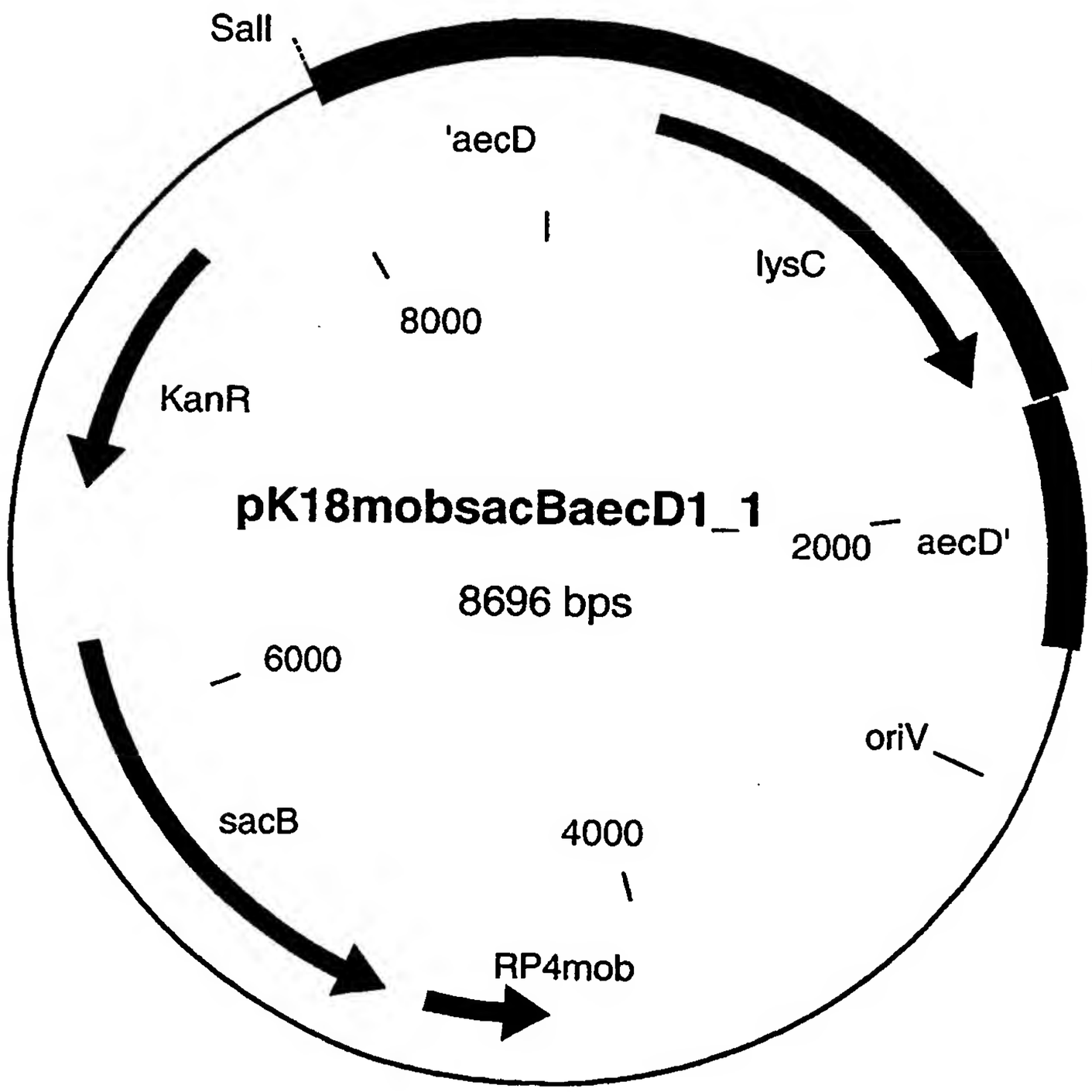
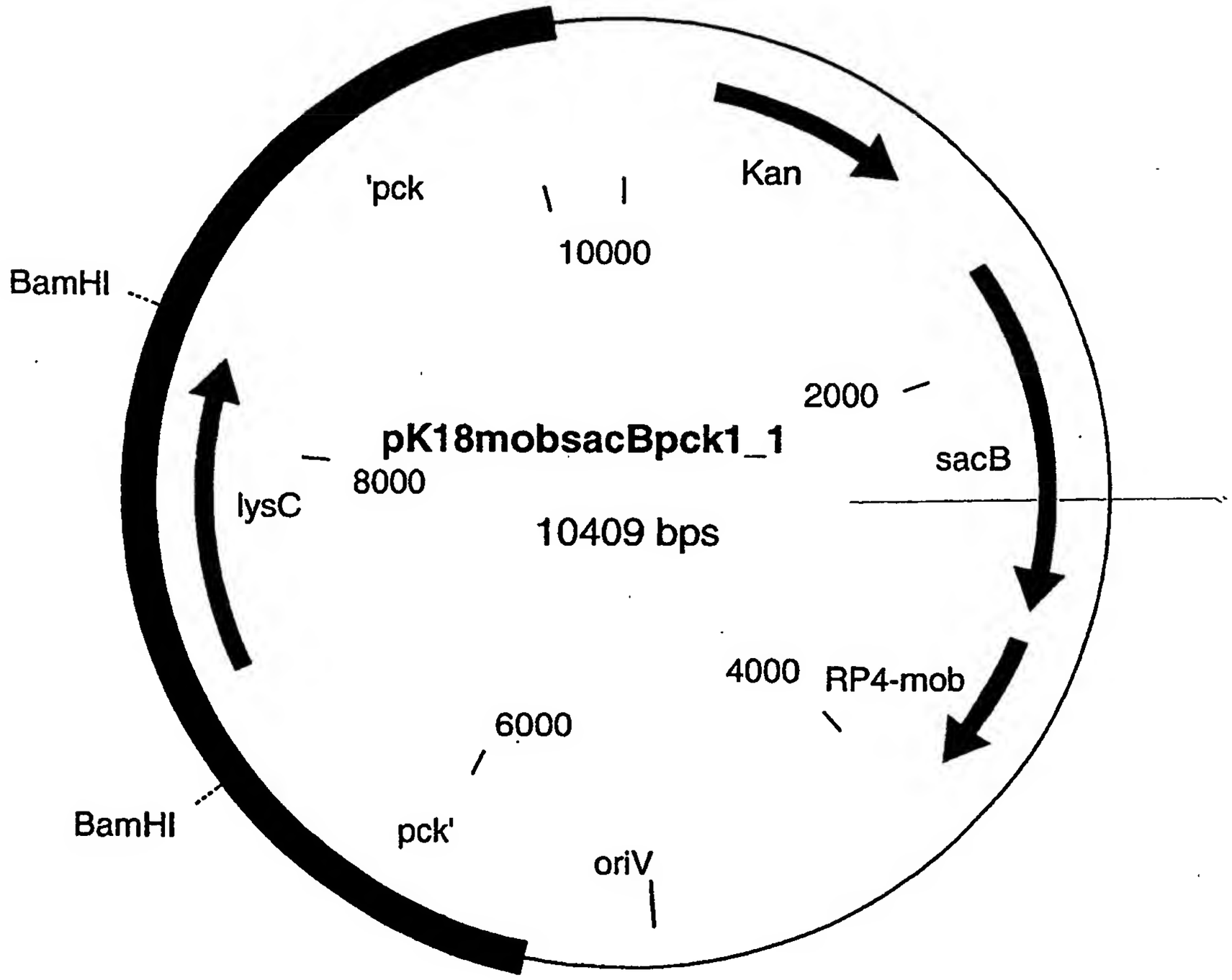


Figure 3: Plasmid pK18mobsacBpck1_1



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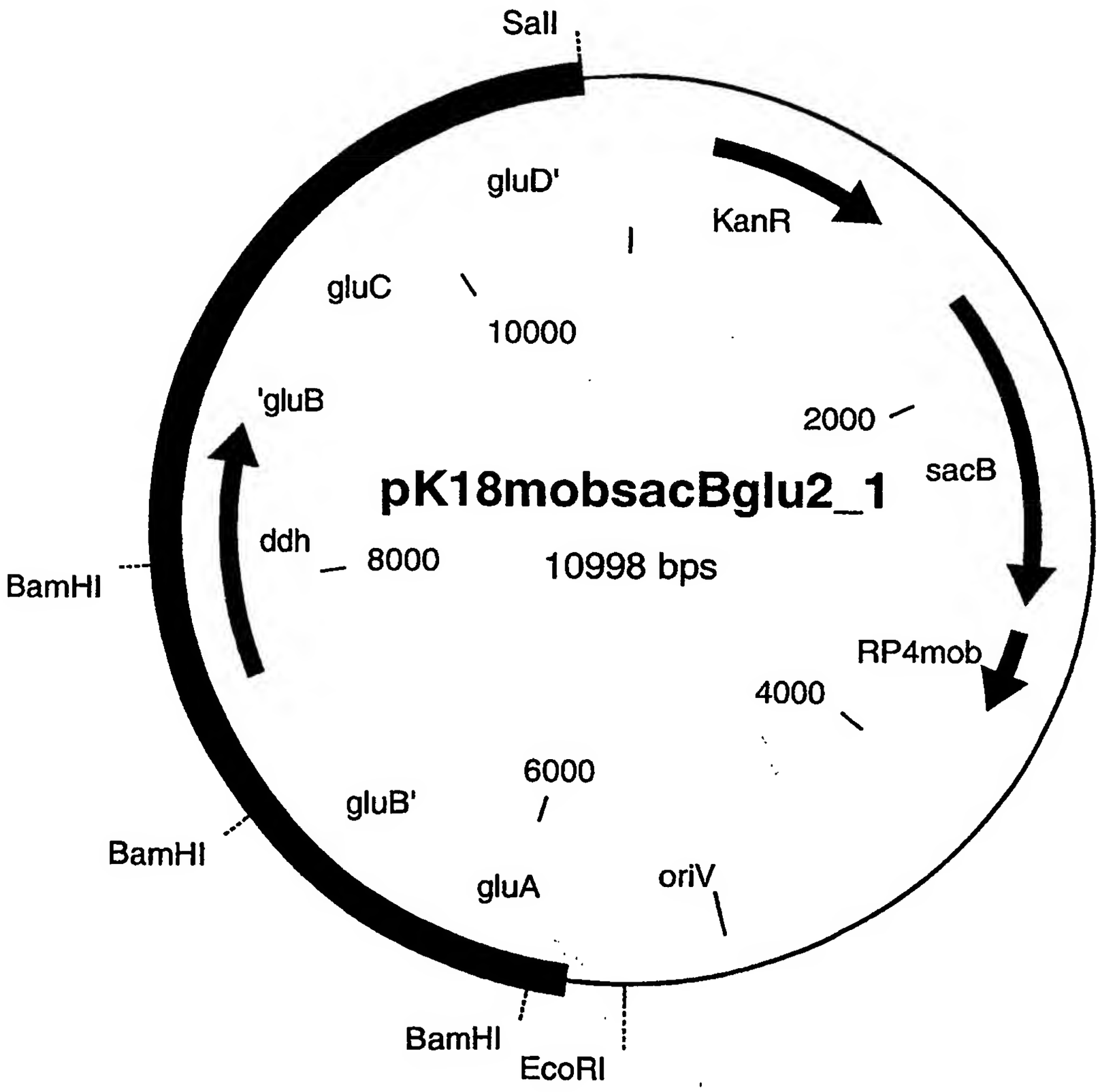

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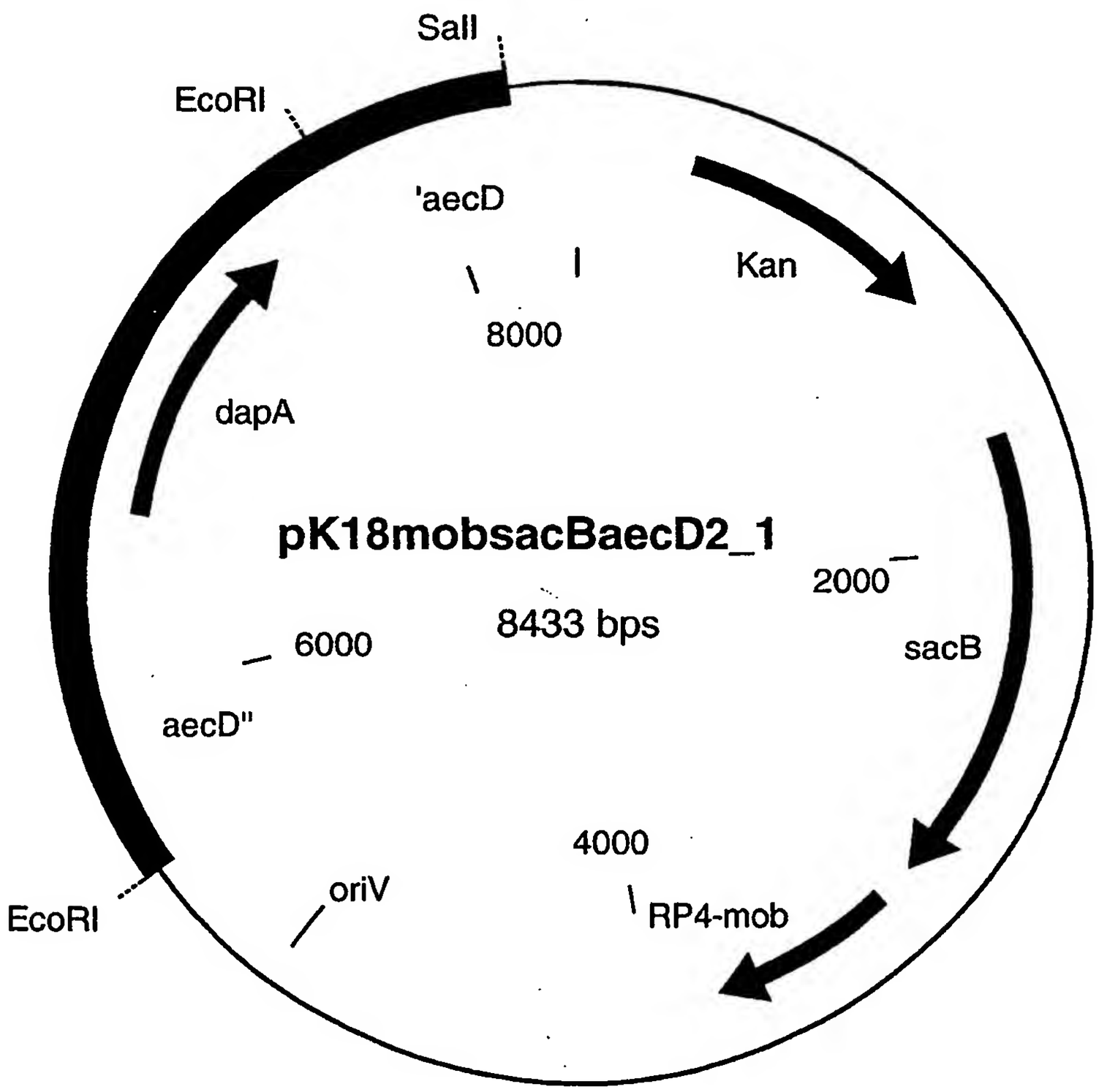
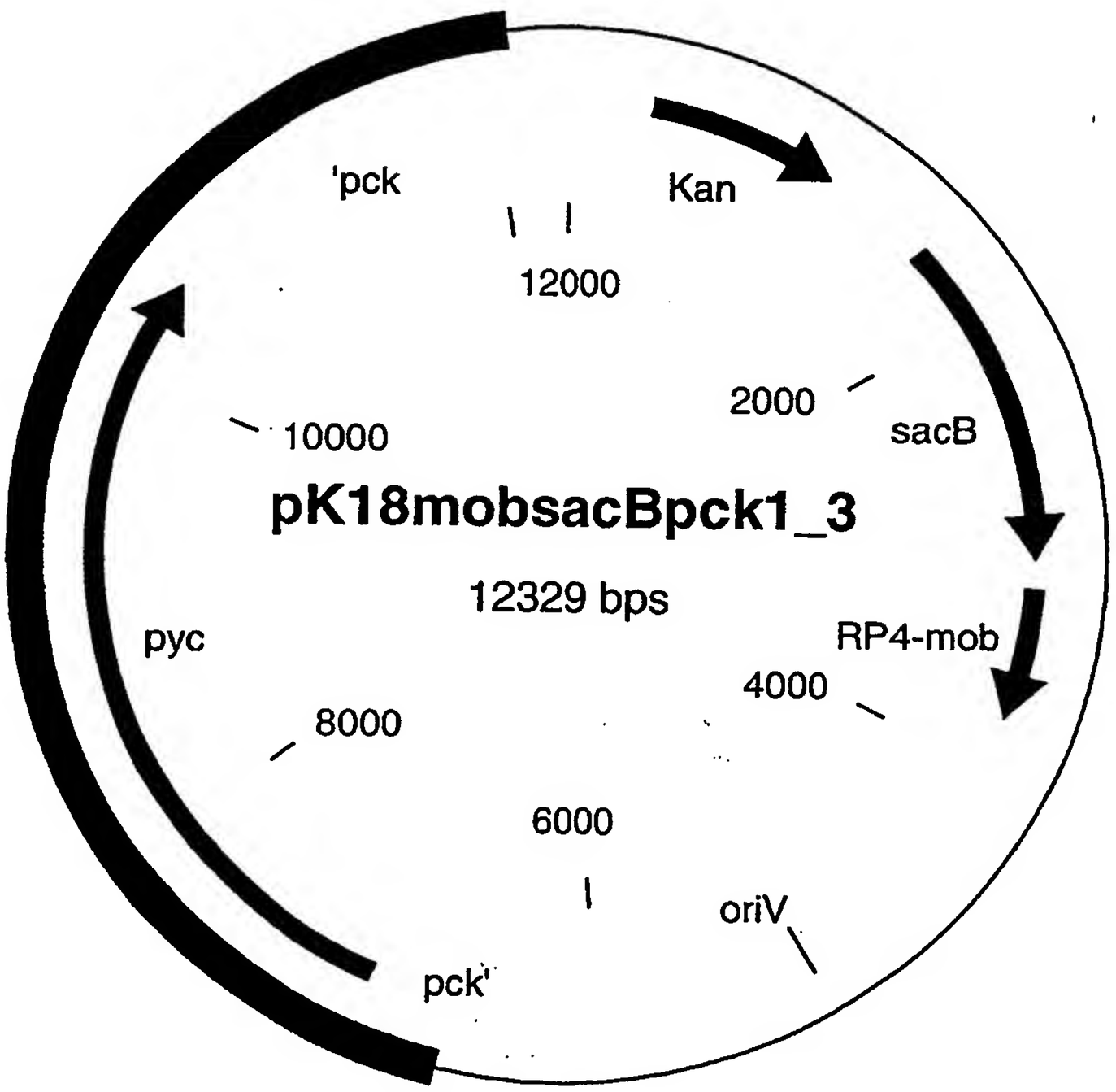


Figure 6: Plasmid pK18mobsacBpck1_3



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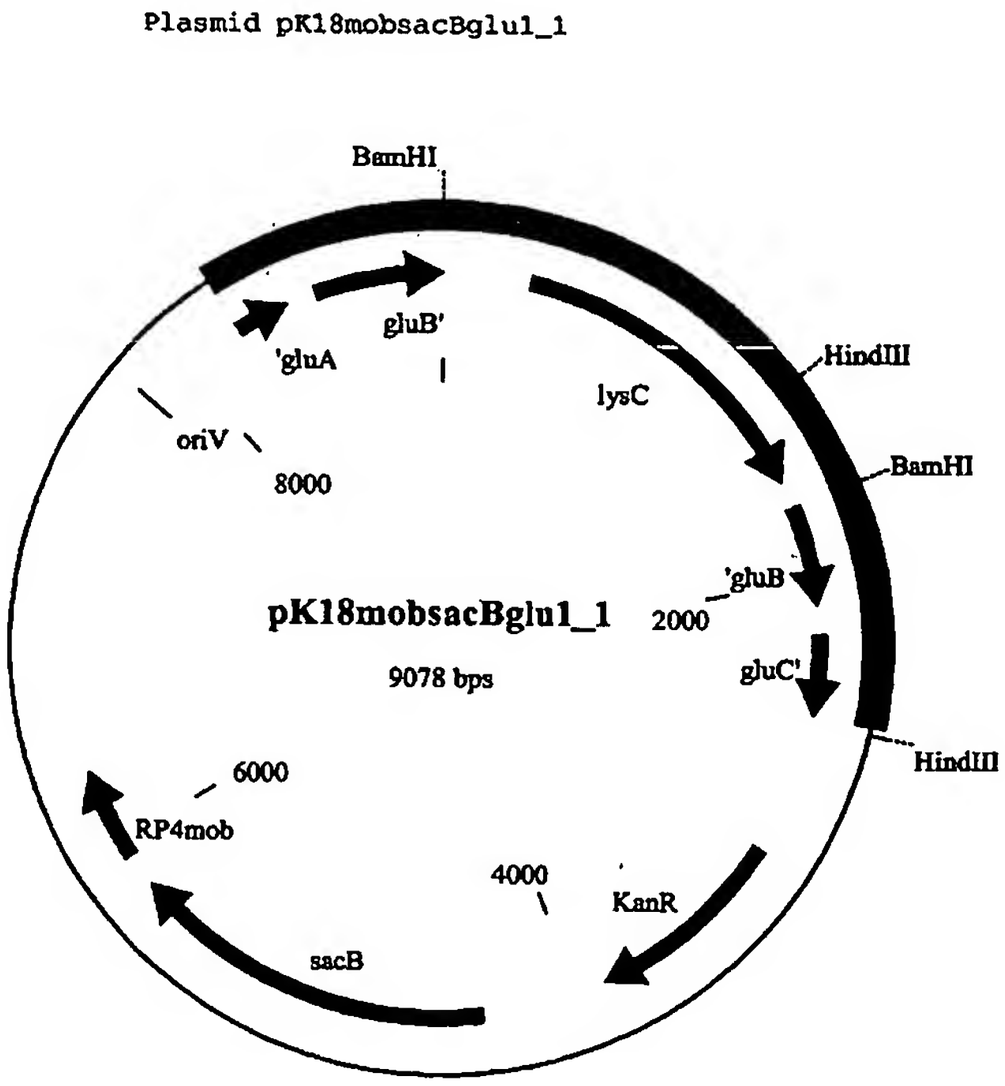
(74) Common Representative: **DEGUSSA AG**; Intellectual Property Management, Patents And Trademarks, Location Hanau, P.O. Box 13 45, 63403 Hanau (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

[Continued on next page]

(54) Title: PRODUCTION OF L-LYSINE BY GENETICALLY MODIFIED CORYNEBACTERIUM GLUTAMICUM STRAINS



(57) Abstract: The invention relates to coryneform bacteria which have, in addition to at least one copy, present at the natural site (locus), of an open reading frame (ORF), gene or allele which codes for the synthesis of a protein or an RNA, in each case a second, optionally third or fourth copy of this open reading frame (ORF), gene or allele at in each case a second, optionally third or fourth site in a form integrated into the chromosome and processes for the preparation of chemical compounds by fermentation of these bacteria.

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European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)*

— *of inventorship (Rule 4.17(iv)) for US only*

Published:

— *with international search report*

(88) Date of publication of the international search report:
18 December 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/08464

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/52 C12N15/53 C12N15/54 C12N15/60 C12P13/08
C12N1/21 //(C12P13/08,C12R1:15)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, SEQUENCE SEARCH, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KRONEMEYER, W. ET AL.: "Structure of the gluABCD Cluster Encoding the Glutamate Uptake System of Corynebacterium glutamicum" JOURNAL OF BACTERIOLOGY, vol. 177, no. 5, March 1995 (1995-03), pages 1152-1158, XP002935147 cited in the application abstract page 1154; figure 2 page 1157, column 1, line 9 - line 13 --- -/--	1-38,40

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 July 2003	08 08. 2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuchs, U

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/08464

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ISHINO, S. ET AL.: "Nucleotide sequence of the meso-diaminopimelate D-dehydrogenase gene from Corynebacterium glutamicum" NUCLEIC ACIDS RESEARCH, vol. 15, no. 9, 11 May 1987 (1987-05-11), page 3917 XP001056569 cited in the application the whole document	1-37
A	EP 1 094 111 A (DEGUSSA-HÜLS AG) 25 April 2001 (2001-04-25) cited in the application the whole document see especially: page 9 -page 10; examples 5,6; tables 2,3	1-37
A	EP 1 067 192 A (DEGUSSA-HÜLS AG; FORSCHUNGSZENTRUM JÜLICH GMBH) 10 January 2001 (2001-01-10) the whole document see especially: page 12 -page 13; examples 12-15 and page 19; claims 1,7-9,11-15,19,20	1-40
A	EP 1 108 790 A (KYOWA HAKKO KOGYO CO., LTD.) 20 June 2001 (2001-06-20) cited in the application the whole document see especially: page 224 -page 228; examples 2,3	1-40
A	WO 00 63388 A (KYOWA HAKKO KOGYO CO., LTD.) 26 October 2000 (2000-10-26) cited in the application abstract SEQ ID NOS: 17 and 18 page 24 -page 28	1-40
P,A	-& EP 1 172 437 A (KYOWA HAKKO KOGYO CO., LTD) 16 January 2002 (2002-01-16) the whole document	1-40
A	SCHÄFER, A. ET AL.: "Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum" GENE, vol. 145, no. 1, 22 July 1994 (1994-07-22), pages 69-73, XP001093898 cited in the application the whole document	1-40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 02/08464

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-37
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-37 partially and 38, 40 completely

   Corynebacterium glutamicum strains DSM13994glu::lysC (see example 1.3), DSM12866glu::lysC (see example 1.4) and DSM12866glu::ddh (see example 2.2), a process for the preparation of lysine involving said strains, a process for the production of said strains, plasmid pK18mobsacBglu1_1;

2. Claims: 1-37 partially

   Corynebacterium glutamicum strains DSM12866pck::lysC (see example 1.6) and DSM12866pck::pyc (see example 4.3), a process for the preparation of lysine involving said strains, a process for the production of said strains;

3. Claims: 1-37 partially and 39 completely

   Corynebacterium glutamicum strain DSM12866aecD::lysC (see example 1.8), a process for the preparation of lysine involving said strain, a process for the production of said strain, plasmid pK18mobsacSaecD1_1;

4. Claims: 1-37 partially

   Corynebacterium glutamicum strain DSM12866aecD::dapA (see example 3.2), a process for the preparation of lysine involving said strain, a process for the production of said strain.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-37

Present claims 1-19 relate to coryneform bacteria defined by reference to desirable characteristics or properties, namely "which produce chemical compounds", "wherein these have, in addition to at least one copy, present at the natural site (locus), of an open reading frame (ORF), gene or allele which codes for the synthesis of a protein or an RNA, a second, optionally third or fourth copy of the open reading frame (ORF), gene or allele in question", "at a second, optionally third or fourth site in a form integrated into the chromosome ... and the second, optionally third or fourth site not relating to open reading frames (ORF), genes or alleles which are essential for the growth of the bacteria and the production of the desired compound". Furthermore, claim 37 relates to a process for the production of such bacteria.

The claims cover all coryneform bacteria having these characteristic or properties, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such bacteria. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the bacteria by reference to results to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the Corynebacterium glutamicum strains produced in examples 1.2-4.3.

The same applies to claims 20-36 relating to a process for the preparation of chemical compounds involving such bacteria. Again, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the preparation of lysine as described in example 5.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 02/08464

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP 1094111 A	25-04-2001	DE	19950409 A1	26-04-2001
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